MEMBRANE DYNAMICS OF THE GM$_1$ GANGLIOSIDE -- CHARACTERIZATION OF THE FUNCTIONAL ROLE OF GM$_1$ IN GROWTH REGULATION AND LIGAND-RECEPTOR INTERACTIONS ON LIPID MOBILITY

by

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LIST OF ABBREVIATIONS

1.	AS	Anthroyl Strearic Acid
2.	BCSG	Brain Cell Surface Glycopeptides
3.	BSA	Bovine Serum Albumin
4.	CHOL	Cholesterol
5.	cisPnA	9,11,13,15-cis,trans,trans,cis-Parinaric Acid
6.	c PnA PC	cis-parinaroyl phosphaatidylcholine
7.	DEPC	Dielaidoylphosphatidylcholine
8.	DMPC	Dimystoylphosphatidylcholine
9.	DPE	Dansyl Phosphatidylethanolamine
10.	DPH	Diphenylhexatriene
11.	DPPC	Dipalitoylphosphatidylcholine
12.	DSC	Differential Scanning Calorimetry
13.	ESR	Electron Spin Resonance
14.	GLC	Gas-Liquid Chromatography
15.	NMR	Nuclear Magnetic Resonance
16.	NS	Nitroxide Strearic Acid
17.	OC	Octadecyl carbocyanine
18.	ONS	Octadecyl naphthylamine sulfonate
19.	P	Perylene
20.	PC	Phosphatidylcholine
21.	TLC	Thin Layer Chromatography
22.	t PnA	9,11,13,15-all trans-Parinaric Acid
23.	t PnA GC	all-trans-Parinaroyl Glucocerebroside
24.	trans PnA	9,11,13,15-all-trans- Parinaric Acid

25. trans PnA GC all-trans-Parinaroyl Glucocerebroside

AN ABSTRACT OF A MASTER'S THESIS

ABSTRACT

As plasma membrane components of many cells, gangliosides have been shown to serve as membrane receptors for various biologically active substances. Furthermore, gangliosides have been demonstrated to modulate the effects of various growth factors. Even though gangliosides are capable of modulating the effects of growth factors such as platelet-derived growth factor and epidermal growth factor, they do not bind to these growth-affecting molecules. We have examined the interaction of gangliosides with molecules which inhibit the growth of cultured animal cells.

Growth-inhibitory glycopeptides have been purified and characterized from bovine cerebral cortex cells. These glycopeptides are capable of inhibiting both protein synthesis and cell division in normal cells. Mouse LM cells have no detectable gangliosides within their plasma membranes and are refractory to the inhibitor. Incubation of exogenous GM_1 gangliosides with the LM cells confers sensitivity to the inhibitor upon the cells. The sensitization of cells to the inhibitory action of the glycopeptides implies that GM_1 may act as a membrane receptor or receptor modulator for these molecules. Elucidation of the functional role played by GM_1 in this growth-regulatory process was analyzed by both *in situ* and *in vitro* ligand-receptor binding assays to detect binding of inhibitor to gangliosides. In cell binding assays, it was shown that incubation of exogenous gangliosides with these cells, though capable of sensitizing them

to the growth inhibitor, did not result in an increase in binding of the inhibitor to the cells. Furthermore, various *in vitro* ligand-receptor binding assays were performed using the cholera toxin-GM_1 ligand receptor model. These studies also demonstrated the inability of the growth inhibitor to bind to GM_1 gangliosides. In addition, protease digestion of cells able to bind the growth inhibitor significantly decreased inhibitor binding. This implies that a membrane protein was involved in the growth inhibitor-membrane interaction. Neuraminidase treatment of the same cells was able to enhance slightly the binding of the growth-inhibitory glycopeptides to the cells apparently from an increase in membrane GM_1. These data suggest that membrane ganglioside GM_1 functions in the modulation of the actual membrane receptor for the growth inhibitory glycopeptides.

Hypotheses explaining the molecular mechanism of GM_1 as a modulator of membrane receptor(s) are speculative at best. Progress in the elucidation of the role of gangliosides as membrane receptors has been achieved through studies of the interaction of the enterotoxin of *Vibrio cholerae* with the GM_1 ganglioside. As a lectin-like molecule, cholera toxin has proved to be a useful investigative tool towards the understanding of membrane structure and receptor mobility as well as receptor function. GM_1 ganglioside has been demonstrated to be freely mobile within the lipid bilayer. Thus, GM_1 is capable of interacting with other membrane components such as membrane proteins (receptors) and lipids. Specifically, when GM_1 is

incorporated into model membrane lipid systems, the interaction of GM_1 with the phospholipid(s) results in an increase in membrane order. We examined the effects of ligand-receptor interaction between GM_1 and cholera toxin to elucidate the functional and behavioral role of GM_1 as a membrane receptor. By employing fluorescence polarization and light scattering, we were able to detect lectin-induced agglutination of liposomes containing GM_1. In addition, glycolipid clustering events within these model membranes resulted in a disordering of the membrane lipids. Such events can significantly alter membrane dynamics which in turn can lead to changes in membrane permeability or cell surface function.

Chapter I.

LITERATURE REVIEW

INTRODUCTON

I. MOLECULAR MEMBRANE BIOLOGY

Biological membranes are organized assemblies consisting mainly of lipids and proteins. Membranes act as highly selective permeability barriers containing specific molecular pumps and gates. In addition, membranes give cells their individuality by separating them from their environment. Membranes serve to control the social interactions between cells and between cells and various biologically active substances in the cell's environment by specific membrane receptors. These receptors receive external stimuli as well as generating chemical or electrical signals for cell-to-cell communication.

Due to the immense diversity of protein and lipid molecules within intact plasma membranes, assignment of biological function to their various components would be an insurmountable task. To simplify the problem, model lipid or reconstituted membrane systems of known lipid and protein composition are employed. Data obtained from such analyses permit definition and assignment of function to the particular membrane constituents.

The current working hypothesis of cell membrane structure is the fluid mosaic model popularized by Singer and Nicholson (1), whereby both protein and lipid are dynamic in nature. Moreover, the fluidity, flexibility, and general physical properties of the plasma membrane are determined by the chemical composition and

physical state of the lipid(s) present. For example, lipids undergo thermal phase transitions, called gel-liquid crystalline transitions, in which the acyl chains of the lipids change from ordered and rigid to disordered and fluid (2,3). This lipid fluidity imparted membranes by lipids has been demonstrated to perform a functional role in several membrane processes such as membrane transport (4,5); activity of membrane bound proteins (6); intercellular communication (7); cellular development (8); and cellular transformation (9,10).

The mobility of membrane components has been examined by various techniques. Physical methods which involve the introduction of a molecular probe into the membrane include: nuclear magnetic resonance (NMR) (11,12); electron spin resonance (ESR) (13,14); differential scanning calorimetry (DSC) (15); and fluorescent polarization (16,17), (for reviews, 18-22). All have advantages and disadvantages. The greatest disadvantage of these spectroscopic techniques is the perturbation of the membrane induced by the probe (16,17).

In the studies described below, we used a pair of linear polyene fatty acid probes 9,11,13,15-cis, trans, trans, cis-octadecatetraenoic acid and its all-trans isomer, 9, 11, 13, 15-all-trans-octadecatetraenoic acid (cis-parinaric acid and trans-parinaric acid, respectively) (See figure 1). These two probes were developed and defined spectroscopically as to orientation and location within the plasma membrane by Sklar, et

al. (23). These two isomers absorb and fluoresce in unique spectral regions (290-325 nanometers and 420 nanometers, respectively) and as such, their fluorescence is not obscured by intrinsic chromophores such as the aromatic amino acids and porphyrins (23, 24). Cis-, and trans-parinaric acid (cis-PnA and trans-PnA, respectively) when incorporated into the membrane are linear and similar enough to naturally occurring components that the degree of perturbation is minimal. Cis-PnA inserts into the membrane and exhibits no preference for solid or liquid state of the lipids. However, trans-PnA when incorporated into the membrane prefers solid phase lipids. Furthermore, the perturbing influences these semi-intrinsic probes are minimal relative to other fluorescent probes (23, 24).

Due to their amphiphatic nature, phospholipids will spontaneously reassociate in an aqueous environment to form bilayers. This self assembly makes biophysical studies on membrane mobility possible (25). The distribution and asymmetry displayed by the phospholipids in a liposome are dependent upon the thermodynamic stability of the constituents (25, 26). Thus, the distribution of phospholipids within a liposome are dependent upon the size of the headgroup, and the packing (degree of unsaturation) of the fatty acid acyl chains (26).

II. THE STRUCTURE AND FUNCTION OF MEMBRANE GANGLIOSIDE

Of the major membrane components, glycolipids, and in

particular, gangliosides, have been shown to interact with various biologically active factors such as, bacterial toxins (9, 27-32); glycoprotein hormones (33-38); viruses (39-41); interferon (39, 41); and possibly growth regulatory glycopeptides (42-44). On the basis of these data, one possible function of membrane gangliosides is to serve as membrane receptors or modulators of other membrane receptors within the plasma membrane. Gangliosides have also been shown to play a role in cellular interactions and differentiation, in cell growth control (oncogenic transformation), and in immune recognition (10). However, the molecular details of ganglioside functions are still speculative due to the lack of information on their organization within the plasma membrane and their surface properties (45).

Gangliosides are amphipathic containing both hydrophobic and hydrophilic regions. They comprise a family of negatively charged acidic glycolipids that are characterized by the presence of at least one sialic acid residue (N-acetylneuraminate or N-glycolylneuraminate). It has been speculated that the negative charge conferred upon the molecule by the sialic acid is important in their function as cell surface receptors for soluble ligands and cellular components. In all gangliosides, one sialic acid is linked to the 3-position of a galactose residue (47). The structure of the common ganglioside species, monosialoganglioside (GM_1) is illustrated in figure 2. GM_1 has a five sugar oligosaccharide containing a single sialic acid resiude. This

hydrophilic head group, which protrudes into the extracellular environment is linked to the hydrophobic sphingosine portion of the ceramide. The carbohydrate side-chain is synthesized via stepwise addition of sugar residues. The sugars are donated by UDP-activated derivatives of the sugars (48).

The receptor function of membrane gangliosides is not clearly understood. One hypothesis states that they exist as monomers within the fluid membrane matrix and ligand binding leads to clustering and subsequent internalization (49-51). An alternative scheme proposes that they initially exist as clusters and binding of biologically active materials causes dispersion reclustering elsewhere in the membrane (52). The receptor function of membrane gangliosides is probably not analogous to that of protein receptors; additionally, they components are probably not as effective a receptor as are glycoproteins. Their receptor function, however is best explained by the dynamic behavior of the gangliosides within the outer leaflet of the plasma membrane. The intrinsic mobility of a ganglioside within the outer leaflet of the plasma membrane is defined by several parameters including the head group structure of the ganglioside species, by the order of the other membrane components, the concentration of a particular ganglioside within the membrane, and the interaction of the ganglioside with a ligand. For example, the mobility of a ganglioside might be decreased by increasing the concentration of that molecule, an effect which may result from the aggregation of

the glycolipids. Further, ligand-induced mobility increases significantly at low concentrations of the ligand molecule (52, 53). However, at higher or saturating concentrations of ligand, ganglioside mobility is greatly reduced (52-56).

Progress in elucidation of the role of gangliosides as membrane receptors has been achieved by studies of the interaction of the enterotoxin of Vibrio cholera with GM_1 (31, 57, 58). Cholera toxin is an oligomeric protein consisting of two major subgroups, A and B with an aggregate molecular weight of 84,000 daltons. There are five B subunits per toxin molecule and one A subunit per t: molecule (59). The B subunit, or cholergenoid, with a molecular weight of 15,000 per unit, is responsible for binding the toxin to the GM_1 membrane receptor. The A subunit is a dipeptide of 28,000 daltons linked by disulfide bridges. It is the A subunit which confers biological activity upon the toxin molecule; this toxicity is dependent upon the B subunit for interaction with cell membranes. The A subunit activates adenylate cyclase in a reaction involving nicotinamide adenine dinucleotide (NAD). This results in greatly enhanced levels of cyclic adenosine 3, 5-cyclic monophosphate (cAMP) (60, 61). The adenylate cyclase is activated irreversibly (59).

The five subunits of B provide it with pentavalent-like properties. Due to the pentavalent nature of the B subunit of cholera toxin behaves analogously to a lectin (53). Lectins are multivalent carbohydrate-binding proteins and can be isolated from

various plants and invertebrates. Each lectin is specific for a certain carbohydrate and because of their multivalent nature will cause agglutination of receptor molecules by crosslinking the surface carbohydrates of adjacent molecules (26). The same phenomenon is also observable in liposomes (26). Thus, lectins have a useful application for understanding membrane structure and mobility as well as receptor function.

III. HYPOTHESES TO BE TESTED

A. DISTRIBUTION OF GANGLIOSIDES WITHIN THE PLASMA MEMBRANE--GANGLIOSIDES EXIST AS MONOMERS IN BIOMEMBRANES

It has been demonstrated (62) that limited concentrations of exogenous gangliosides can be incorporated into lipid bilayers. Using these stable, GM_1-containing bilayer systems several investigators (62,63) have been able to show that the incorporation of GM_1 into the lipid bilayer increases the stability of the membrane. However, no single model membrane system encompasses all the biological features of the cellular plasma membrane.

The first data on the mobility of GM_1 was by Riedler (64). Using the technique of fluorescence photobleaching recovery and a fluorescent analog of GM_1, Riedler examined the lateral mobility of GM_1-like molecule within 3T3 mouse fibroblast plasma membranes. Furthermore, he found that the GM_1 probe was unaffected by cytoskeletal poisons; by crosslinking membrane

glycoproteins with either lectin or antibodies; or by transforming the fibroblasts with SV40. Additional observations by Riedler showed that cholera toxin was capable of patching the GM_1 probe, an effect which again was unaltered by cytoskeletal poisons. These data imply that GM_1 is capable of diffusing freely throughout the plasma membrane and that the GM_1 receptors do not appear to be localized in discrete domains within the bilayer.

Recent work in our laboratory (51), using parinaric acid fluorescent probes in unilamellar phosphatidylcholine vesicles, has demonstrated that increasing amounts of GM_1 incorporated into liposomes, results in an increase in the order of the membrane lipid. This stability in the membrane, induced by GM_1, is more evident in phosphatidylcholine vesicles with longer and saturated acyl chains. This has been demonstrated with both dipalmitoyl- and dimyristoyl-phosphatidylcholine (DPPC and DMPC, respectively). In phosphatidylcholine vesicles comprised of fatty acids with saturated 18-carbon acyl chains, there is a decrease in the membrane order as compared to the shorter saturated species of lipid.

The increase in membrane order observed in these liposome systems is due to interaction between the GM_1 ganglioside and the lipid within the vesicles. GM_1 incorporation into unilamellar phosphatidylcholine (PC) vesicles induces a phase separation at temperatures above the phase transition of PC vesicles alone. This phenomenon, as detected by fluorescence polarization, holds

for all PC vesicles independent of acyl chain length of the fatty acids. Further since GM_1 exhibits no phase transition within the temperature range of these experiments, the data imply that GM_1-PC interaction increases membrane order.

Other studies which also suggest that sphingolipids exist in discrete domains came from Lee, *et al.*, (52) who used gangliosides with spin-labeled oligosaccharides to measure anistropy of various GM_1/PC vesicles. From their data, Lee, *et al.*,(52) were able to conclude that the size of the oligosaccharide head group played a role in the dispersion of gangliosides within the phospholipid bilayer and their dynamic state within the membrane.

All of these data taken together provide the compelling reasons for pursuing the investigation of the dynamic behavior of GM_1 within the plasma membrane. The behavior of ligand-receptor interaction was analyzed as the the effects of ligand binding on the molecular motion of the GM_1 ganglioside within experimental membranes. Specifically, the interactions of the ligands, cholera toxin and peanut lectin, with dielaidoylphosphatidylcholine (DEPC) were studied. The results of these experiments will be helpful in elucidating both the functional and behavioral role of GM_1 as a membrane receptor.

B. FUNCTIONAL ROLE PLAYED BY GANGLIOSIDES IN GROWTH REGULATION -- EXAMINATION OF RECEPTOR FUNCTION FOR GROWTH-INHIBITORY GLYCOPEPTIDES

Gangliosides not only function as membrane receptors for biologically active substances and as modulators of protein and glycoprotein membrane receptors, they have been shown to function in the regulation of cell growth and cellular interaction (10). Dramatic changes in glycolipid composition and metabolism associated with oncogenic transformation suggest a specific role for membrane glycolipids in the regulation of cell growth and cellular interaction. Two general types of changes in ganglioside composition of the plasma membrane are observed in transformed cells which produce tumor-distinct glycolipids. The deletion of complex gangliosides in transformed cells may arise from a block in the stepwise synthesis of the gangliosides leading to an accumulation of precursor structures. Alternatively, these simpler glycolipids may arise from altered activity of glycosyltransferases which leads to the production of new glycolipids atypical to those normally expressed in the untransformed cell (For review, see ref. 10).

The possible role for glycolipids in cell growth control has been investigated by various approaches. Exogenous incorporation of gangliosides into the cellular membrane from culture media (65-68), or addition of growth regulatory substances and growth modulators (36-38,43, 44), or antiglycolipid antibodies (69) have all been utilized. These treatments are capable of greatly affecting cell growth, behavior, morphology, and saturation density. Typically, normal cells in the presence of gangliosides

show increased cellular adhesiveness and reduced saturation density, whereas, transformed cells mimic normal cells and show significant enhancement of glycolipid synthesis.

Changes in glycolipid composition have also been associated with cellular interaction and differentiation. Studies have demonstrated that cell contact induces enhanced glycolipid synthesis, thus, implicating glycolipids as the basis of contact inhibition of cell growth (For review, see ref. 10). It has been proposed that oncogenesis and differentiation are related processes in that, in each response, there is a dynamic continum in the alterations of the composition of the cell surface structure. Obviously, the involvement of gangliosides in these processes is a subject of intense interest.

Recently, growth-inhibitory glycopeptides have been purified and characterized from bovine and murine cerebral cortex cells (78-83). These brain cell surface glycopeptides (BCSG) contain two inhibitory fractions of molecular weights 12,000 and 15,000 daltons that are capable of inhibiting the protein synthesis and cell division in normal cells in a dose dependent manner. The growth inhibition by these molecules occurs during protein translation inhibiting elongation of nascent polypeptide chains. The inhibitor is capable of exerting its effects at physiological (ng/ml) concentrations. Inhibition is non-lethal, reversibile, and has not been shown to be either species or tissue specific. Cells without membrane gangliosides or undetectable levels of the

GM$_1$ ganglioside, remain refractory (78-82). For example, mouse LM cells which have no detectable gangliosides within their plasma membrane are refractory to the inhibitor. However, preincubation of LM cells with GM$_1$ ganglioside confers sensitivity to the inhibitor (43). This sensitization of LM cells to the inhibitory action of the glycopeptides implies that the GM$_1$ ganglioside may act as a membrane receptor or serve as a modulator of the receptor for the growth-inhibtory glycopeptides.

In similar analyses, mouse 1316 fibrosarcoma cells were shown to be refractory to the growth inhibitor (43). The unresponsive nature of these transformed cells was associated with an absence of the GM$_1$ ganglioside detected by thin layer chromatography (TLC). Furthermore, TLC was employed to demonstrate the ability of the 1316 cells to incorporate GM$_1$ under appropriate conditions. Fibrosarcoma cells preincubated at 0 ^{o}C for two hours with GM$_1$ incorporated the ganglioside and became sensitive to the growth inhibitor. However, if after preincubation of 1316 cells with GM$_1$ at 0 ^{o}C the cells were returned the cells to 37 ^{o}C, the cells once again became refractory to the inhibitor. The concomitant disappearance of GM$_1$ in 131o cells after incorporation was suggested to reflect the cells' transformed phenotype.

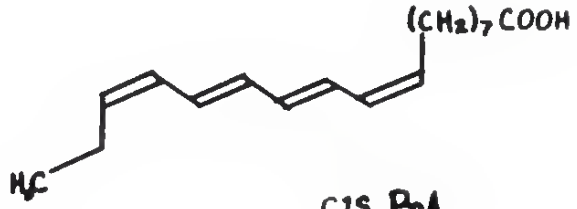
The interaction of BCSG with the cell at the level of the plasma membrane is required for biological activity. The possibilty exists that the monosialoganglioside, GM$_1$, is a BCSG receptor or modulator of BCSG activity. In the present study, therefore, the

elucidation of the functional role of GM_1 in the sensitization of cells to the growth-regulatory processes of BCSG were investigated by employing the cholera toxin-GM_1 ligand-receptor system as a reference model in various ligand-receptor binding assays. These studies enable us to discern whether the inhibitor binds to GM_1 or whether GM_1 serves merely to modulate the function of the actual receptor.

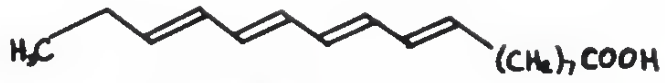
Figure 1: Line drawings of parinaric acid probes characterized by L. Sklar (PhD Dissertation, 1976).

A. 9, 11, 13, 15-cis, trans, trans, cis- parinaric acid;

B. 9, 11, 13, 15-all-trans-parinaric acid.

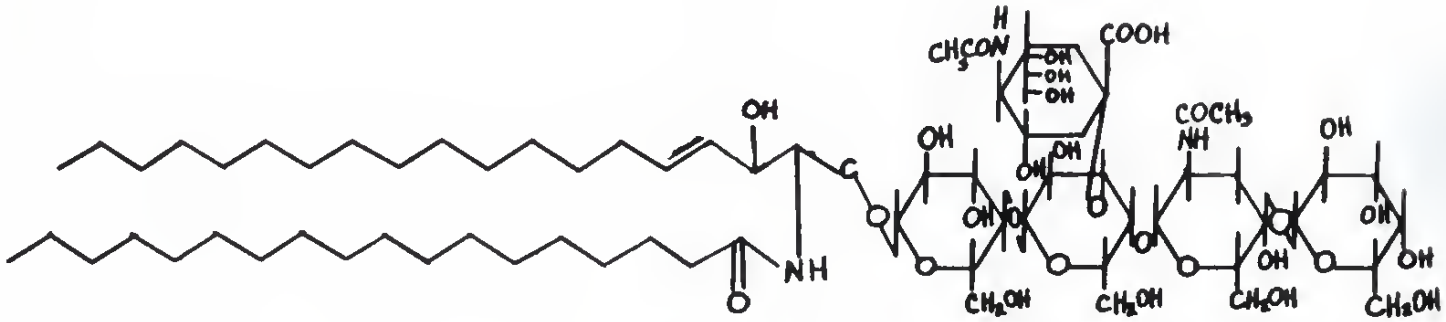


cis PnA



trans PnA

Figure 2: Structure of Monosialoganglioside, GM_1


OH
NH
O
COOH
OH
OH
O

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Chapter II.

LIGAND BINDING TO GM-1 GANGLIOSIDES -- EFFECTS ON LIPID MOBILITY

INTRODUCTION

Plasma membranes provide selectively permeable barriers between cells and their environment. They control the amount and nature of the substances that pass between or enter cells. They are composed primarily of lipids and proteins. The lipids form a fluid matrix in which the proteins are embedded (1). As such they interact with each other, playing different but coordinated roles in the functioning of the membrane.

In addition to lipid and protein, plasma membranes contain carbohydrates. Membrane carbohydrates of eukaryotic cells are covalently linked to either protein or lipid molecules (glycoproteins and glycolipids, respectively). Glycoproteins and glycolipids associate and interact with one another and comprise a membrane structure called the glycocalyx. The physiological behavior and function of the glycoconjugates contained within this matrix are largely dependent upon their lateral mobility and topographical distribution within the membrane bilayer and with the type of phospholipid associated with them (2-5).

Both glycoproteins and glycolipids are found distributed throughout the plasma membrane. They have been shown to serve as cell surface receptors for various hormones, for cellular recognition markers, and for viruses (6-10). Thus, glycoproteins and glycolipids via their surface carbohydrate moieties are multifaceted components of the plasma membrane that perform

specific roles in cellular communication, differentiation and growth regulation.

In comparison to glycoproteins, relatively little is known about the physical properties, organization, or receptor functions of membrane glycolipids, particularly, the glycosphingolipids. Recent studies indicate that glycolipids can modify various cell surface functions (11-14). They may, like phospholipids, modulate the activities of certain membrane proteins via direct interaction (2, 15). Additionally, their function and organization may contribute to the overall structural properties of the bilayer matrix through interaction with membrane phospholipids (16, 17). Furthermore, gangliosides have been demonstrated to be receptors for bacterial toxins (18-21), peptide hormones (15, 22-25), viruses (26-28), neurotransmitters (29), and lectins (30, 31). It is likely that the variation in oligosaccharide composition and structure of these glycosphingolipids and the variation in their patterns of occurrence on various cell types permits the broad receptor diversity of these molecules. However, in most cases, no detailed models exist which can explain the mechanism(s) by which ganglioside receptors communicate biological stimuli to the cell (32-35). Is it the interaction of the ganglioside with other molecules of the glycocalx, that is, other glycolipids or glycoproteins through which gangliosides communicate with the cell?

Recent studies have examined the influence of gangliosides on

the physical properties of cell membranes. Specifically, properties of ganglioside/phospholipid mixtures in multi- and unilamellar dispersions of the monosialoganglioside, GM_1, and dipalmitoylphosphatidylcholine (DPPC) have been examined (36-41). It was been shown that addition of glycosphingolipids to Phosphatidylcholine (PC) vesicles results in an increase in lipid order (36, 37, 41) indicating a role for gangliosides in domain formation and stabilization within the plasma membrane. Additionally, it has been demonstrated that GM_1 is miscible with PC in lipid dispersions up to 25-30 mole % GM_1 (36, 41). However, the nature of GM_1/PC at mixtures containing more than 30 mole % is yet to be completely resolved (36, 37, 41). Furthermore, when GM_1/PC model membranes are fluid, that is, at temperatures above the lipid phase transition of the PC species, GM_1 gangliosides are randomly distributed throughout the bilayer. However, below the transition temperature, some investigators believe that clustering of the GM_1 gangliosides occurs within the lipid matrix (39). In contrast, in our laboratory (41) and in others (36, 37) evidence has been obtained which suggests that gangliosides are miscible in PC in both the solid and fluid phases.

Despite these model membrane studies on the molecular organization of GM_1 and its interactions with various phospholipid species, the role played by GM_1 as a membrane receptor and its effects on membrane structure remains largely unexplained. GM_1 has been demonstrated to serve as a membrane receptor for cholera

toxin (18, 42-44), wheat germ agglutinin (31, 45), and the galactose-specific lectin of Ricinus communis beans (30, 46) in both cellular membranes and in GM_1-containing liposomes. The latter, has been found to be a useful model system for the investigation of agglutination of cells by lectins.

We report here our studies on the effects of lectin binding on receptor motion and distribution using GM_1/dielaidoylphosphatidylcholine (DEPC) as a model membrane system. Specifically, the interactions of cholera toxin and peanut lectin (47, 48) with GM_1-containing DEPC unilamellar liposomes will be examined to ascertain:

(1) The effects of ligand binding on the mobility of GM_1 receptor molecule.

(2) The effects of ligand binding on the mobility of the phospholipid.

(3) The affects of the physical structure of the phospholipid on binding of ligand to receptor.

To address these questions, the agglutination of GM_1/DEPC vesicles will be monitored by following changes of both fluorescence depolarization by 90° light scattering (49-51), and fluorescence polarization ratios to measure the membrane dynamics of these agglutination events. Our preliminary hypothesis is that ligand binding reduces the mobility of gangliosides in fluid model membranes and increases mobility in solid membranes. Additionally, we propose that ligand binding increases the mobility of phosphatidylcholine in fluid model membranes, but

decreases mobility in solid membranes.

EXPERIMENTAL PROCEDURES

MATERIALS

Ganglioside GM$_1$ was obtained from Supelco (Bellefonte, PA). 1, 2-dielaidoyl-3-sn-phosphatidylcholine (DEPC), cholera toxin, and peanut lectin were obtained from Sigma Chemical Company (St. Louis, MO) and were used without further purification. Both the cholera toxin and peanut lectin were reconstituted with fluorescence buffer (10mM HEPES, 50mM HCl, pH 7.2) to a final concentration of 100 nmoles/ml. All solvents used were of spectral grade (Fisher). *Cis*- and *trans*-parinaric acids were contributed by Dr. R. Simoni, Department or Biological Sciences, Stanford University, Stanford, CA. The N-parinaroyl spingolipid probe, N-*trans*-parinaroyl glucocerebroside (tPnAGC) was synthesized and characterized in our laboratory (41). The 2-*cis*-parinaroyl phosphatidylcholine (cPnA PC) probe was the generous gift of Dr. R. Welti, Department of Biochemistry, Kansas University Medical Center, Kansas City, KS.

PREPARATION OF LIPOSOMES

Liposomes of dielaidoylphosphatidylcholine (DEPC) and DEPC/GM$_1$ (80/20, mole/mole) were prepared by the ethanol injection technique of Batzri and Korn (52) as modified by Welti and Silbert (53). Solutions of phospholipid and glycolipid in chloroform were

dried down under a continous stream of N_2. The lipid film was resuspended in 20 ml of absolute ethanol and warmed to 45-50 °C. This lipid solution was injected by means of a 25 ml Hamilton syringe into 4.0 ml of degassed fluorescence buffer at 45-50 °C. This is an ethanol-water concentration of approximately 0.5% (v/v) and about 400 nmoles total lipid per 4.0 ml. These unilamellar liposomes were then analyzed by flourescence polarization spectroscopy and light scattering methods as described below. Previous analysis of liposomes prepared similarly in our laboratory have been shown to contain 95-100% of the added phosphatidylcholine and 98-100% of the added ganglioside (41).

FLUORESCENCE POLARIZATION SPECTROSCOPY

Liposomes, prepared as described above, were placed in a 2.0 ml quartz cuvette in the thermostatted, temperature controlled cuvette of a Spex Fluorolog Spectrofuorometer. Excitation wavelengths were 320 nm for tPnA and its glycosphingolipid derivative, tPnA GC, and 325 nm for cPnA and its phospholipid derivative, cPnA PC; emission was monitored at 420 nm for both probes. Blank (fluorescence intensity without added probe) emission at the lowest scan temperature was recorded for both parallel and perpendicular emission (using vertically polarized light for excitation), and the sample was then heated to the highest scan temperature. Blanks were again recorded, and the parinaric acid probe was added to the same cuvette. For experiments using parinaroylglycolipid or phospholipid probes, the

blanks were recorded using buffer alone. The liposomes were prepared by resuspending the ganglioside/phosphatidylcholine mixtures in an ethanolic solution of parinaroyl phospholipid for injection into buffer as described above. Fluorescence emission parallel and perpendicular to the excitation was monitored as the sample was cooled at a rate of 0.75 ^{o}C / minute, using a Neslab Water Bath and Linear Temperautre Programmer. Data were analyzed using the computer smoothing program previously described (54). No corrections were made for scattering depolarization (50), since all samples had absorbances of less than 0.100 at the excitation wavelengths. Data obtained by heating the mixtures rather than by cooling were equivalent, implying that the structures formed are equilibrium mixtures of the components. Additionally, liposomes stored for several days at 4 ^{o}C gave identical fluorescence polarization thermotropic profiles when compared to freshly made liposomes, implying that the structures were stable with regard to time (41).

OPTICAL STUDIES

The interaction of cholera toxin and peanut lectin with GM_1-containing DEPC and DEPC liposomes was monitored by fluorescence depolarization and 90 ^{o} light scattering (49-51). The 90 ^{o} light scattering was monitored at a wavelength of 450 nanometers. The effects of ligand binding to DEPC and DEPC/GM_1 (80/20, mole/mole) on membrane dynamics was determined from fluorescence polarization spectroscopy. Free fatty acid probes

were incorporated after liposome preparation by direct addition to the liposome suspension after taking background measurements. The final probe to lipid ratio was 1:200 in all cases. Fluorescence polarization and light scattering were measured on a Spex Fluorolog Spectrofluorometer. Titration studies of cholera toxin (1 nmole / 10 ml or 100 nmoles / ml) and peanut lectin (1 nmole / 10 ml, or 100 nmoles / ml) were performed by the direct addition of ligand to the liposomes contained within the quartz cuvette. The ligand solution was uniformly dispersed in the liposome suspension by gentle continous stirring.

RESULTS AND DISCUSSION

The molecular motions of N-trans parinaric acid (tPnA), N-trans parinaroyl glucocerebroside (tPnA GC), and N-cis parinaroyl phosphatidylcholine (cPnA PC) fluorescent lipid probes in dielaidoylphosphatidylcholine (DEPC) model membranes containing GM_1 gangliosides were studied during titration experiments with cholera toxin and peanut lectin, in order to observe the effects of ligand binding to gangliosides in mixed dispersions of ganglioside and phospholipid. Estimates of rotational motion at 5.0 +/- 0.5 ^{o}C and 35.0 +/- 0.5 ^{o}C were determined from fluorescence polarization ratios, while the binding of lectin was monitored by changes in light scattering.

Fluorescence polarization allows the monitoring of the rotation exhibited by a fluorescent membrane probe under a variety of

conditions (for example, temperature range or lipid content). Thus, data obtained from fluorescence polarization studies can be used to interpret not only the motion, but the order of the lipid species under investigation as well. Depending on the type of fluorescent probe employed, an investigator can use fluorescence polarization technique to detect: (1) subtle changes in lateral phase separations of minor lipid species of the membrane (61); (2) phase transition in major lipid species of the membrane (62); and (3) the specific lipid domains preferred by the probe whether in mixed model membrane or in reconstituted lipid-protein systems (63, 64).

Fluorescence polarization spectroscopy has been used to analyze the physical properties of various lipid components in biological and model membranes (55-60). These studies followed the molecular motions of a relatively large fluorophore, 1, 6-diphenyl-1, 3, 5-hexatriene (DPH). While these fluorescence studies have provided relevent information on the structural integrity of membranes, DPH as a membrane probe causes significant membrane perturbation which affects the results obtained (65). This is not to imply that the use of fluorescence polarization spectroscopy is not a useful and advantageous technique for analyzing lipid interactions. Rather, it is to suggest that ideal membrane probes to carry out such studies should minimize membrane perturbation in order to minimize difficulties in interpretation of the data.

For these studies we have employed derivatives of the

naturally-occurring cis- and trans-parinaric acid molecular probes first prepared and characterized by Sklar (65). Unlike many other fluorescent membrane probes these molecules do not perturb the membrane structure and are biological in origin. Because they resemble integral membrane components they can be readily incorporated into either eukaryotic or procaryotic phospholipids (66, 67) as well as into artificial and biological membrane systems (63, 68). Cis-parinaric acid has been found to resemble an unsaturated fatty acid and is incorporated into the fluid lipid layer. In contrast, trans-parinaric acid resembles a saturated fatty acid and is preferentially located within the solid lipid phases of the membrane. Thus, by employing these two isomeric fatty acid probes, it is possible to detect both lipid phase transitions (with cPnA) and lipid phase separations (with tPnA) (61).

For these experiments, tPnA and tPnA GC were employed to detect lipid phase separations during the binding of lectins to DEPC/GM$_1$ (80/20, mole/mole) liposomes. In binary mixtures of phospholipids, tPnA exhibits a preference for solid phase lipids (54, 61). The tPnA GC sphingolipid probe exhibits a preference for solid phase glycolipids. In DEPC/GM$_1$ vesicles, by virtue of its resemblence to natural glucocerebroside (GC) it should behave as an analogue of the glycolipid component. Therefore, tPnA GC is likely to be associated with the ganglioside fraction of our liposome system. However, the cPnA PC probe should detect the

DEPC transition in the phosphatidylcholine (PC) domain, based on its resemblence to naturally-occurring PC (54, 62). Furthermore, cPnA PC has been demonstrated to exhibit a preference for the fluid lipid phase in mixed PC membranes regardless of head group (63). Therefore, the cPnA PC probe should be enriched in the DEPC enriched domains in our system (Table 1).

LIPID INTERACTIONS BETWEEN GM_1 AND DEPC IN MODEL SYSTEMS

Pure GM_1 in aqueous suspension form spheroidal micelles (69-71) and does not display a thermotropic phase transition over the temperature range from 12-83 °C (36). Pure monoglucocerebroside, likewise, has no detectable phase transition over a similar temperature range (10-75 °C) (72). In contrast, the major model membrane component, DEPC, in aqueous solutions forms extended lamellar bilayers (73). In our laboratory, DEPC has been demonstrated to exhibit a melting temperature between 10-13 °C (41). DEPC membranes containing up to 30 mole % GM_1 retain the bilayer or lamellar structure (70). The type of lamellar structures formed, is determined primarily by the balanced interactions between their head groups, which are normally repulsive, and between their hydrophobic tail groups (74, 75). Incorporation of more than 30 mole % ganglioside in PC results in a marked physical change in structure leading to the formation of mixed micelles (69-71). Results from our laboratory correlate with these observations (41). Upon addition of increasing amounts of GM_1 to PC liposomes (up to 25-30 mole %) an increase in the

transition temperature of the PC species was observed. In GM_1/PC mixtures, the phase transition of the PC, detected by fluorescence polarization of cPnA will shift from 10-13 °C for pure DEPC up to 20-22 °C for PC/GM_1 mixtures of 75/25 mole/mole. Thus, incorporation of GM_1 into PC bilayers results in a higher fluorescence polarization ratio which indicates a more ordered PC lipid phase. The phase transition temperatures obtained in this particular study also demonstrate an increase in lipid order as exhibited by the change of the melting temperature of DEPC. The melting temperature of DEPC shifts from about 17 °C in DEPC liposomes to 23 °C for DEPC/GM_1 liposomes (80/20, mole/mole) (Figure 4). These data give us confidence that our fluorescence polarization studies can be interpreted based on previous fluorescence (41) and calorimetric (36, 37) data.

LIGAND INTERACTION WITH LIPOSOMES CONTAINING GANGLIOSIDES -- DEMONSTRATION OF AGGLUTINABILITY

DEPC/GM_1 (80/20 mole/mole) liposomes were used as a model system for the investigation of agglutination of liposomes by lectins. Binding studies were done at 5.0 +/- 0.5 °C and 35.0 +/- 0.5 °C by adding increasing concentrations of either of two lectins, namely, cholera toxin and peanut lectin to different preparations of liposomes containing one of our three fluorescent probe species (for discussion of probes refer to above).

When lectin was added to the DEPC/GM_1 liposomes increases in the

90^{o} light scattering were observed (Figure 5). The increase in 90^{o} light scattering represents agglutination of liposomes. In both cholera toxin and peanut lectin the binding event was rapid. This was to be expected for the cholera toxin which exhibits a K_d value of 1.1×10^{-9} M in purified liver membrane preparations and 4.6×10^{-10} M for fat cells (43). The K_d of peanut lectin as not been reported. However, incubations at the various concetrations of peanut lectin for up to 90 minutes did not alter the amount of detected binding. The same was observed for cholera toxin. Thus, the observed increases in light scattering represent a rapid agglutination event of DEPC/GM$_1$ vesicles and the formation of liposome clusters by these lectins. However, the addition of cholera toxin to DEPC/GM$_1$ vesicles agglutinated the DEPC/GM$_1$ liposomes appreciably more (5-6 times higher scattering depolarization values) than did the peanut lectin (Tables 4 and 5). Furthermore, these agglutination events were demonstrated to be specifically related to ligand interaction with the GM$_1$ receptor molecules because neither lectin was able to agglutinate liposomes composed solely of DEPC. Additionally, the extent to which DEPC/GM$_1$ liposomes were agglutinated was increased as the molar ratio of lectin to GM$_1$ was increased (receptor in excess; Table 2, and Figure 5). During these binding events, agglutination of DEPC/GM$_1$ liposomes was not temperature dependent. However, agglutination of the DEPC/GM$_1$ liposomes was slightly better at lower temperatures (Tables 4 and 5).

In support of our data, similar interactions have been reported between the lectin from *Ricinus communis* (30) and wheat germ agglutinin (WGA) (31, 76) with PC liposomes containing GM_1 gangliosides. *Ricinus communis* lectin reacts specifically with the terminal galactose of GM_1 (46, 77, 78). While WGA has been demonstated to interact with the sialic acid residue on GM_1 (45, 79). In neither study was the lectin able to bind to vesicles containing only PC. However, both lectins cause significant agglutination of PC liposomes containing GM_1. This observed formation of liposome clusters by lectin-ganglioside interaction in ganglioside/PC model membranes has been suggested to resemble cell agglutination (30). Thus, our approach of employing PC vesicles containing gangliosides should prove useful as a model for furthering the investigation of cellular agglutination mechanisms.

The lesser extent to which peanut lectin was able to bind to GM_1 in the model membranes may be a result of steric hinderance from the sialic acid residues present on GM_1 molecules. Peanut lectin has been demonstrated to bind to mouse, rat, guinea pig, and human lymphocytes, but only after neuraminidase treatment. (Neuraminidase hydrolyzes sialic acid residues from glycoproteins and glycolipids.) Treatment of neuraminidase-treated lymphocytes with α-galactosidase significantly decreases the agglutination of the cells upon addition of peanut lectin (48). These data imply that peanut lectin reacts specifically with terminal galactose

residue and its ability to interact with a receptor is reduced by the presence of sialic acid on the glycolipid or glycoprotein. However, we cannot directly test this hypothesis, since the single sialic acid in GM_1, is very resitant to neuraminidase hydrolysis (43,84). From our data, however, peanut lectin was still observed to agglutinate DEPC/GM_1 liposomes. Increases in agglutination during titration, on the average of 1.3 fold higher, were obtained, but only at the lower binding temperature. Lectin dependent increases in light scattering were not detectable at 35.0 °C. Furthermore, it can be argued for the binding of both cholera toxin and peanut lectin to GM_1 that the surface changes resulting from lectin binding one receptor molecule on one liposome may be sufficient to permit interaction of the lectin with available receptors on adjacent liposome vesicles. This resulted in the agglutination of DEPC/GM_1 liposomes we observed for situations of receptor excess.

LIGAND INTERACTION WITH LIPOSOMES CONTAINING GANGLIOSIDES -- INFLUENCES ON MEMBRANE FLUIDITY

Agglutination of ganglioside-containing liposomes is dependent upon changes in both the topological distribution of the ganglioside receptor and membrane fluidity. Studies examining the topographics of glycolipids as membrane receptors are being pursued (3-5, 81, 82). However, the molecular aspects of membrane fluidity in agglutination processes involved in cellular adhesion during development, differentiation, oncogenesis, and

ligand-receptor interactions is still speculative. To address the issue of membrane fluidity in cell agglutination, we chose to examine the affects on membrane fluidity of lectin-ganglioside complexes and cluster formation in DEPC/GM$_1$ model membranes.

From our fluorescence polarization data (data summarized in Table 3), cholera toxin was shown to disorder DEPC/GM$_1$ bilayers at temperatures below the phase transition (5.0 +/- 0.5 ^{o}C) of DEPC/GM$_1$ (80/20 mole/mole). This disordering of membrane lipids was interpreted from the observed decreases in fluorescent polarization ratios exhibited by each of three membrane probes employed. Specifically, the disordering changes in membrane structure of the DEPC component was detected with both the trans-parinaric acid (tPnA), the trans-parinaroyl glucocerebroside (tPnA GC), and the cis-parinaroyl phosphatidylcholine (cPnA PC) probes. The tPnA probe should be monitoring the molecular motion of all major membrane components. Based on previous data, the cPnA PC probe should monitor the molecular motion of the DEPC. The trans-parinaroyl glucosylcerebroside (tPnA GC) sphingolipid probe probably associates mainly with the minor GM$_1$ fraction. No effect on membrane fluidity was detected for titration studies performed: (1) with cholera toxin or peanut lectin to vesicles composed of DEPC at either temperature; (2) with cholera toxin to DEPC/GM$_1$ at temperatures (35.0 +/- 0.5 ^{o}C) above the melting temperature (T_m) of DEPC/GM$_1$ (80/20, mole/mole); or (3) with peanut lectin to DEPC/GM$_1$ at either low or high binding temperatures.

We considered the possibility that the rapid increase in light scattering (i.e., the rapid binding of lectin to GM_1 as presented above) may be depolarizing the fluorescence emission. Previous investigators have reported that scattering can significantly depolarize fluorescence emission in both biological and model membranes (51). In particular, Lentz, *et al.*, (50) have reported that appreciable increases inlight scattering by membranes affects polarization ratios. By employing the method described by Teale (49) we were able to determine that the fluorescence polarization ratios presented in Table 3 were not affected by light scattering. The change in optical density of our liposome suspensions before and after addition of up to 5.0 nanomoles of either cholera toxin or peanut lectin was very small and we could not detect changes in the polarization ratio upon dilution of the sample.

Reidler (83) examined the mobility of selected fluorescent lipid analogs on cell membranes. Through the interactions of various effector molecules with cell membranes, he was able to demonstrate the "free" mobility of GM_1 within the membrane; that is, GM_1 mobility is not the result of cytoskeletal interactions. In experiments performed with cholera toxin to examine glycolipid mobility via a fluorescent glycolipid probe, Reidler was able to demonstrate toxin dependent clustering of GM_1 analogs in cell membranes. He concluded that the head groups of bound GM_1 molecules, behaved as a "non-free draining unit." Furthermore,

the formation of the fluorescent patches caused a reduction in the mobility of the glycolipid-associated membrane probe. However, Reidler was unable to address the interactions of glycolipid with phospholipids in his studies of cholera toxin-GM_1 binding. Furthermore, he was unable to demonstrate the consequences of ligand interaction with ganglioside receptors and on membrane structure. However, with careful selection of fluorescent probes known to partition between solid/fluid lipid phases and to associate within specific lipid fractions, we were able to demonstrate that binding of cholera toxin to GM_1 within DEPC/GM_1 membranes, the ganglioside receptors are agglutinated causing the subsequent disordering of the membrane lipid.

The biological implications of this observation include the possiblity that the interaction of ganglioside receptors can modify the cell membrane. Such events can significantly alter the dynamics of membrane structure. Disordering of membrane lipids by glycolipid clustering events thus can lead to changes in membrane permeability or cell surface function.

In summary, we have demonstrated:

(1) Cholera toxin and peanut lectin agglutinate DEPC/GM-1 model membranes. Furthermore, these agglutination events were specifically related to ligand interaction with the GM-1 receptor molecules.

(2) The extent to which DEPC/GM-1 liposomes were agglutinated was increased as the molar ratio of lectin to GM-1 was increased.

(3) Agglutination of DEPC/GM-1 liposomes did not exhibit a significant temperature dependence, although agglutination was slightly better at the lower binding temperature.

(4) Cholera toxin agglutinated DEPC/GM-1 vesicles better than peanut lectin. Binding by peanut lectin may have been appreciably less than that exhibited by cholera toxin because of steric hinderance by the sialic acid present on GM-1.

(5) Cholera toxin binding to GM-1 within DEPC/GM-1 model membranes causes agglutination of the ganglioside receptors and the subsequent disordering of the membrane lipid. Such an event can significantly alter membrane dynamics which in turn can lead to changes in membrane permeabilty or cell surface function.

Figure 3: Fluorescent membrane probes.

(Diagram adapted for L. Sklar, PhD Dissertation, Stanford University, 1976)

From left to right, upper monolayer:

AS = anthroyl stearic acid

DPE = dansyl phosphatidylethanolamine

OC = octadecyl carbocyanine

trans PnA = all-trans-parinaric acid

trans PnA GC = all-trans-parinaroyl glucocerebroside

From left to right, lower monolayer:

ONS = octadecyl naphthylamine sulfonate

DPH = diphenylhexatriene

ANS = anilino naphthylamine sulfonate

NS = nitroxide stearic acid

P = perylene

cis PnA PC = cis-parinaroyl phosphatidylcholine

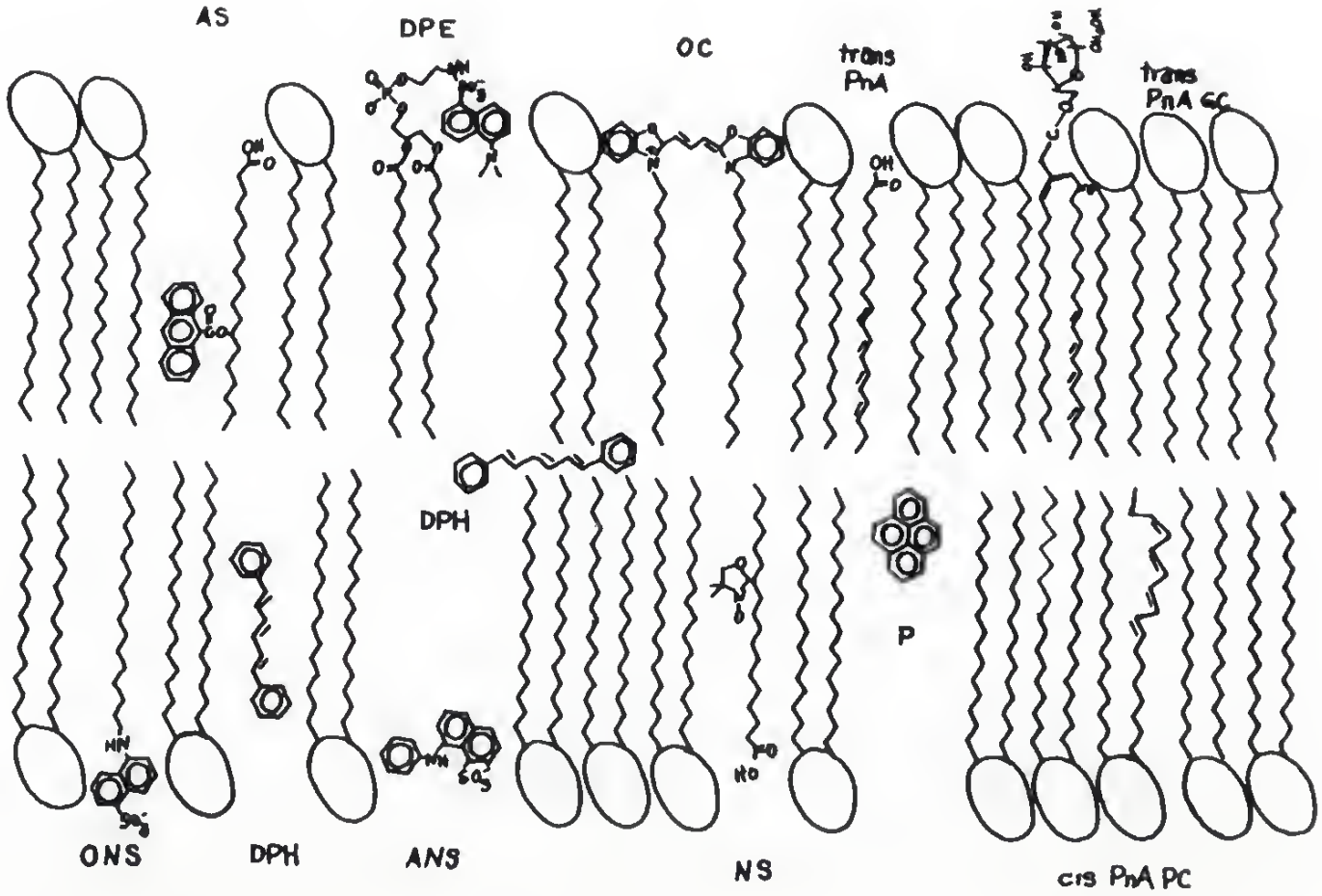
AS
DPE
OC
trans PnA
trans PnA cc
DPH
P
ONS
DPH
ANS
NS
cis PnA PC

Table 1: Fluorescent parinaric acid membrane probes with their membrane counterparts, and their lipid phase and location preferences.

Parinaric Acid Probe	Membrane Counterpart	Lipid Phase Preference	Liposome Fraction Associated With (Location, experimental)
t PnA	Saturated Fatty Acid	solid	Major Membrane Component
t PnA GC	Neutral Gluco-Cerebroside	solid	GM-1 Ganglioside
c PnA PC	Phosphatidyl-Choline	fluid	Dielaidoyl-Phosphatidyl-Choline (DEPC)

Figure 4: Temperature Dependence of tPnA Fluorescence Polarization in Liposomes Containing DEPC and GM_1.

Liposomes were prepared and analyzed as described in the experimental procedures. tPnA (final probe/lipid ratio = 1/200, mole/mole) was added, the liposomes were cooled at a rate of 0.75 °C/min in a Spex Fluorolog spectrofluorometer. Data were collected, blanks were subtracted and smoothed curves were generated as previously described (41). The open circles represent DEPC liposomes and the closed circles represent DEPC/GM_1 (80/20, mole/mole) liposomes. Panel A is a plot of Fluorescence Polarization Ratios versus temperature and Panel B which contains the same data as Panel A, is a plot of a derivative of the fluorescence polarization ratio versus temperature. Similar results were obtained with t PnA, t PnA GC, and c PnA PC fluorescent probes.

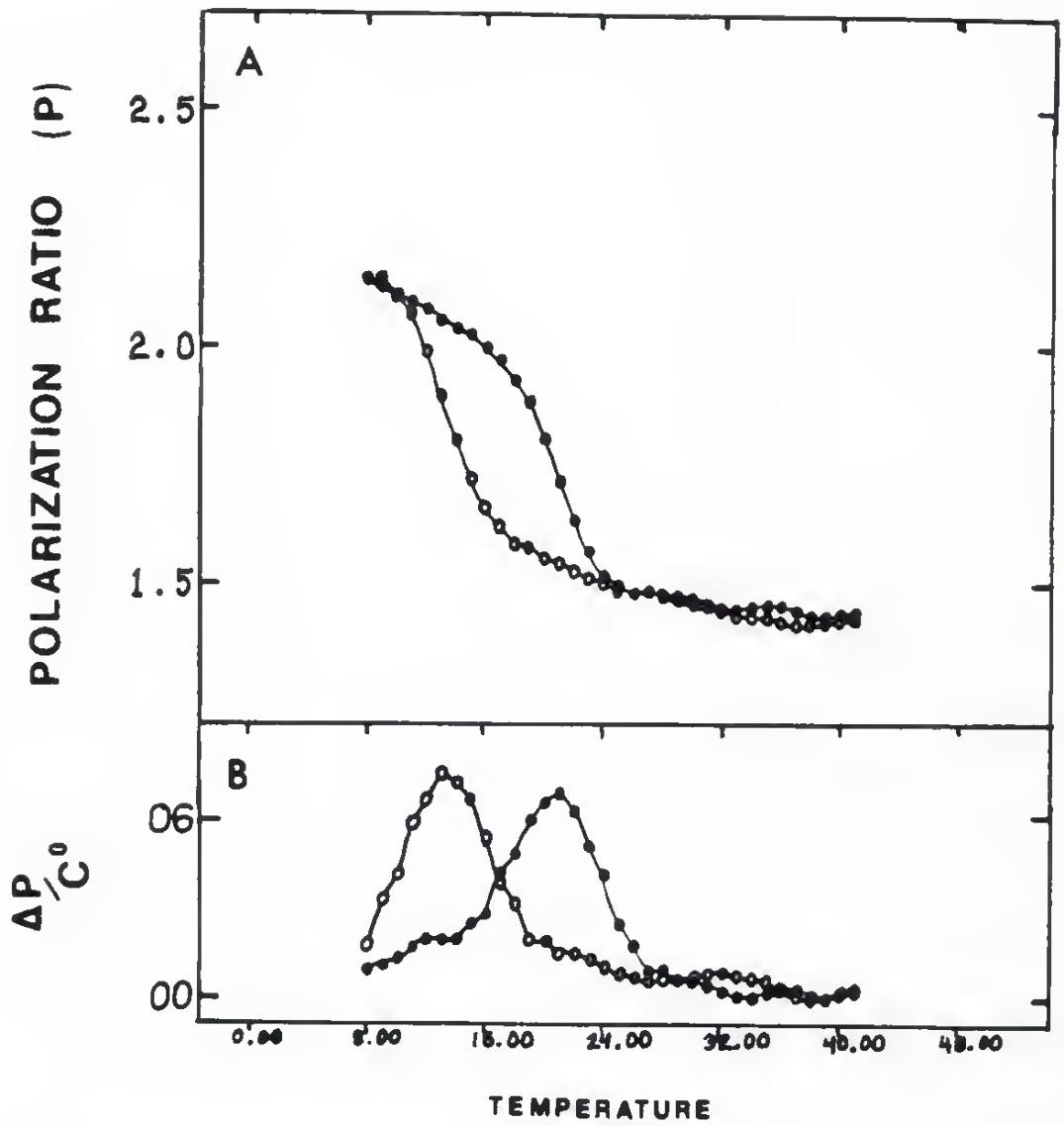
A
B
POLARIZATION RATIO (P)
2.5
2.0
1.5
06
00
0.00
8.00
16.00
24.00
32.00
40.00
48.00
TEMPERATURE

Figure 5: Ligand Interaction With DEPC Liposomes Containing GM-1 Gangliosides -- Demonstration of Agglutinability via 90^{o} Light Scattering.

DEPC/GM-1 (80/20, mole/mole) liposomes were prepared as described in the experimental procedures. Binding studies were done at 5.0 +/- 0.5 ^{o}C and 35.0 +/- 0.5 ^{o}C by adding increasing concetrations of either of the two lectins, cholera toxin and peanut lectin to different preparations of liposomes containing c PnA PC. 90^{o} light scattering was monitored at 450 nm. The open circles represent DEPC liposomes and the closed circles represent DEPC/GM$_1$ (80/20 mole/mole) liposomes. The left panel is the 90^{o} light scattering of liposomes in the presence of cholera toxin and the right panel is the 90^{o} light scattering of liposomes in the presence of peanut lectin. Similar plots of 90^{o} light scattering versus nanomoles of lectin added were obtained at both 5.0 and 35.0 ^{o}C and for all three probes.

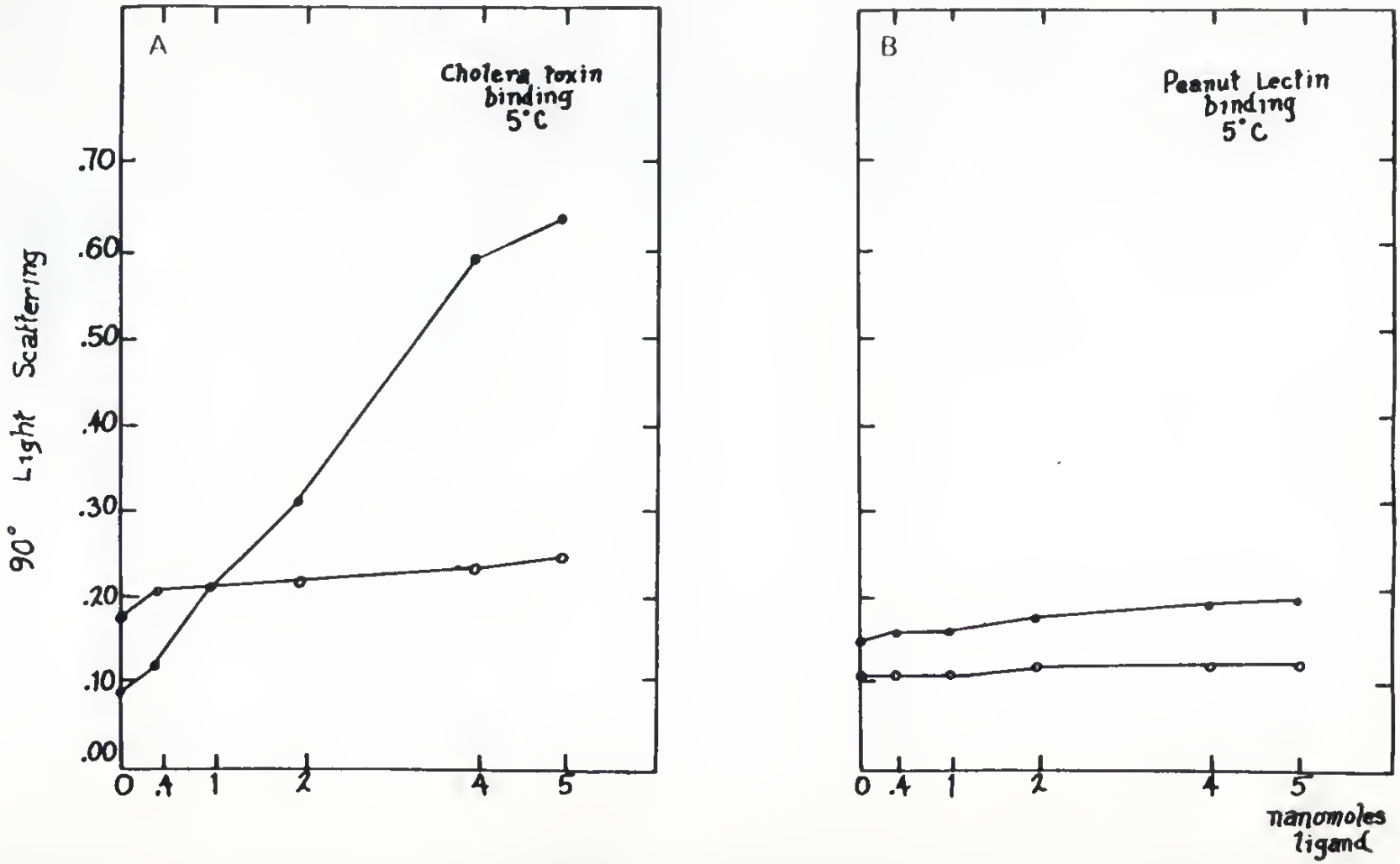
A
Cholera Toxin
binding
5°C
90° Light Scattering
.70
.60
.50
.40
.30
.20
.10
.00
0
.4
1
2
4
5
B
Peanut Lectin
binding
5°C
0
.4
1
2
4
5
nanomoles
ligand

Table 2: Ratios of Cholera Toxin and Peanut Lectin Ligands to the GM-1 Receptor.

Ligand concentrations were 1 nanomole per 10 micoliters (or 100 nanomoles per milliliter).

Ratio of Ligand to GM-1	Ratio of GM-1 to Ligand	Nanomoles of Ligand	Microliliters of Ligand Added
0.00	0	0.0	0.0
0.02	50	0.4	4.0
0.05	20	1.0	10.0
0.10	10	2.0	20.0
0.20	5	4.0	40.0
0.25	4	5.0	50.0

Titration binding studies were performed with receptor excess.

Table 3: Fluoresence Polarization Ratios of Cholera Toxin and Peanut Lectin Binding to Dielaidoylphosphatidylcholine Liposomes With and Without GM-1 Gangliosides.

Ligand	Liposome Composition	Fluorescent Probe Employed	Fluorescence Polarization Ratios Binding at: 5 °C		Binding at: 35 °C	
			Receptor to Ligand Ratio			
			100:0	5:1	100:0	5:1
peanut lectin	DEPC	t PnA	2.02	2.03	1.33	1.39
		t PnA GC	1.96	1.95	1.33	1.38
		c PnA PC	1.76	1.80	1.21	1.19
peanut lectin	DEPC + GM-1	t PnA	2.15	2.10	1.27	1.42
		t PnA GC	2.05	2.01	1.39	1.34
		c PnA PC	1.79	1.88	1.19	1.20
cholera toxin	DEPC	t PnA	1.98	2.01	1.28	1.31
		t PnA GC	1.94	1.95	1.30	1.35
		c PnA PC	1.49	1.48	1.23	1.18
cholera toxin	DEPC + GM-1	t PnA	2.00	1.94	1.37	1.36
		t PnA GC	1.95	1.85	1.31	1.23
		c PnA PC	1.77	1.55	1.24	1.17

Table 4: Data Summary of Peanut Lectin Interaction With GM-1 Gangliosides.

Liposome Composition	Fluorescent Probe	Fluorescence Polarization Ratio 5 C	Fluorescence Polarization Ratio 35 C	90° Light Scattering 5 C	90° Light Scattering 35 C
DEPC (100)	t PnA	NC	NC	NC	NC
	t PnA GC	NC	NC	NC	NC
	c PnA PC	NC	NC	NC	NC
DEPC + GM-1 (80/20)	t PnA	NC	NC	1.75X	NC
	t PnA GC	NC	NC	1.20X	NC
	c PnA PC	NC	NC	NC	NC
			Mean =	1.32X	

NC = No significant change between fluorescence polarization ratios between no ligand and 5:1 receptor to ligand ratio.

Table 5: Data Summary of Cholera Toxin Interaction With GM-1 Gangliosides.

Liposome Composition	Fluorescent Probe	Fluorescence Polarization o Ratio o 5 C	35 C	90° Light Scattering 5 C	35 C
DEPC (100)	t PnA	NC	NC	NC	NC
	t PnA GC	NC	NC	NC	NC
	c PnA PC	NC	NC	1.30X	NC
			Mean =	1.10X	
DEPC + GM-1 (80/20)	t PnA	2.00->1,94	NC	6.14X	4.40X
	t PnA GC	1.95->1.85	NC	6.00X	6.16X
	c PnA PC	1.77->1.55	NC	7.10X	4.50X
			Mean =	6.41X	5.16X

NC = No significant change between fluorescence polarization ratios between no ligand and 5:1 receptor to ligand ratio.

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Chapter III.

INTERACTION OF GROWTH-INHIBITORY BRAIN CELL SURFACE GLYCOPEPTIDES WITH CELLS

INTRODUCTION

The growth inhibitory activity of Brain Cell Surface Glycopeptides (BCSG) has been studied (1-7). It was determined that GM$_1$ monosialoganglioside enhanced the sensitivity of cells to the effects of the inhibitor (7). The possibility that GM$_1$ membrane ganliosides constituted the biological receptors for BCSG was considered. However, even though exogenously incorporated GM$_1$ enhances cellular sensitivity to growth inhibition by BCSG (6), incorporation of GM$_1$ onto cell membranes does not increase the ability of radioiodinated BCSG (^{125}I-BCSG) to bind to the cell surfaces (34). We could not detect ^{125}I-BCSG binding to GM$_1$ using several *in vitro* ligand-receptor binding assays (see chapter 4). Therefore, we concluded GM$_1$ does not serve as a membrane receptor for BCSG. However, GM$_1$ does serve to modulate the biological activity of BCSG (31).

These studies describe procedures used to delineate how the growth inhibitor interactes with the plasma membrane. Calculations based on the binding of ^{125}I-BCSG to 3T3 mouse fibroblast cells imply that there are about 1-2 X 10^4 receptor molecules per cell (31). We have utilized F745 Friend erythroleukemic and AG8 mouse myeloma cells to study the interaction of BCSG with intact cells. These two cell lines were selected because: (1) they bind ^{125}I-BCSG; (2) they proliferate rapidly; and (3) they grow in suspension culture. These characteristics of F745 and AG8 cells make them useful in

experiments designed to determine the identity and characteristics of the plasma membrane components which might be involved in BCSG-membrane interaction.

EXPERIMENTAL PROCEDURES

MATERIALS

GROWTH INHIBITORY GLYCOPEPTIDES (BCSG)

Radioiodinated brain cell surface glycopeptides (^{125}I-BCSG) were the generous gift of Dr. T. C. Johnson, Division of Biology, Kansas State University, Manhattan, KS (1-7).

CELLS

The F745 Friend erythroleukemic cells, provided by Dr. D. Luse (Department of Biochemistry, University of Cincinnati Medical School, Cincinnati, OH) were cultured in modified Eagle's medium (MEM) (Flow Laboratories, McLean, VA) with 5% newborn calf serum (NBCS) (Sterile Systems, Inc., Logan, UT) in 20mM HEPES, glutamine, and nonessential amino acids at 37°C and 5% CO_2. The AG8 mouse myeloma cells were kindly contributed by Dr. G. W. Fortner (Division of Biology, Kansas State University, Manhattan, KS). The AG8s were cultured in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, McLean, VA) containing 10% fetal calf serum (FCS) (Sterile Systems, Inc., Logan, UT), 20mM HEPES, glutamine, and nonessential amino acids at pH 6.8-7.3 in an atmosphere containing 10% CO_2.

ENZYMES

Neuraminidase (8, 9), trypsin (10, 11), alpha-chymotrypsin (11-14), and proteinase K (15) were obtained from Sigma Chemical Company (St. Louis, MO). Cells were enzymatically digested for 40 minutes with either 1.0 unit/ml neuraminidase, 1.0 mg/ml trypsin, 1.0 mg/ml alpha-chymotrypsin, 0.5 mg/ml trypsin and 0.5 mg/ml alpha-chymotrypsin, or 0.25 mg/ml proteinase K prior to performing the cell binding assay (20).

Neuraminidase, prepared from Clostridium perfringes, is a sialidase receptor destroying enzyme, one unit of which has been determined to liberate 1.0 umole of N-acetyl-neuramic acid (NANA) per minute at pH 5.0 at 37 ^{o}C (9). Trypsin, from beef pancreas, specifically hydrolyzes peptides, amides and esters at lysine and arginine carboxyl bonds (11-14). Alpha-chymotrypsin, also purified from beef pancreas, is a member of a group of proteolytic enzymes derived from chymotrypsinogens A and B. The chymotrypsins have relatively broad specificity, hydrolyzing aromatic amino acids, amides, and amino acyl moieties (11-14). Proteinase K causes nonspeicific hydrolysis of proteins (15).

METHODS

CELL BINDING ASSAY

The cells were counted and 2.0 X 10^{6} cells were used per replicate. The appropriate volume of cells was pelleted by

centrifuging at 1000 rpm for five minutes. The cell pellets were resuspended and washed twice in 2.0 ml Hanks BBS, pH 7.2, (16). For binding of ^{125}I-BCSG to cells 800 ul total volumes are used (four replicates were contained within one reaction tube). The binding assays were carried out in Falcon polystyrene 12 X 75 mm test tubes pretreated 24-48 hours at 4 ^{o}C with 5% Bovine Serum Albumin (BSA). At the end of the incubation period, 200 ul aliquots were removed. The reaction halted by adding the 200 ul sample into 2.0 ml cold Hanks BBS (pH 7.2) and centrifuged at 1000 rpm for five minutes. The cell pellets were washed a second time with 0.5 ml Hanks BBS (pH 7.2) and the amount of ^{125}I-BCSG bound was determined by counting gamma emission in a Packard Multi-Prias Gamma Counter.

RESULTS AND DISCUSSION

A number of studies have demonstrated that exposure of cells to glycolipids modifies various cells surface functions (21-24). Glycosphingolipids added exogenously to cells in culture are incorporated into the plasma membranes where they have been shown to be capable of inhibiting cell growth and modify growth behavior (22, 25-27). Specifically, GM$_1$ and GM$_3$ gangliosides have been demonstrated, in serum-free medium, to inhibit cell growth when incorporated into cell membranes (28, 29). Furthermore, GM$_1$ and GM$_3$ were shown to be capable of inhibiting growth factor-stimulated mitogenesis, possibly through modulation of growth factor receptor function. One mechanism proposed for this

modulation of membrane receptors is via inhibition of tyrosine phosphorylation stimulated by growth factor binding (29). We have observed enhancement of growth inhibition by the growth-inhibitory BCSG upon incorporation of exogenous gangliosides and have speculated that gangliosides modulate BCSG-receptor function (31). However, before analyses of glycolipid-receptor interactions can be pursued, the membrane components involved in BCSG-membrane interaction must be identified and characterized.

ANALYSIS OF ^{125}I-BCSG INTERACTION WITH CELLS

DEMONSTRATION OF MEMBRANE GANGLIOSIDES

The F745 Friend erythroleukemic cells and AG8 mouse myeloma cells selected for this study have been demonstrated by the ganglioside isolation procedure of Irwin and Irwin (17) and by thin layer chromatography (TLC) to contain membrane gangliosides (data not shown). F745 and AG8 membrane gangliosides chromatographed on TLC plates were developed with resorcinol, a reagent which specifically interacts with the sialic acid present within the ganglioside head groups (18). F745 cells contained predominantly GM_1 and GD_{1a} ganglioside species, as well did the AG8s.

^{125}I-BCSG BINDING TO CELLS

^{125}I-BCSG has been demonstrated to bind to cells in culture (31). We demonstrate here that ^{125}I-BCSG is also capable of

binding to both F745 and AGB cells. This binding has been demonstrated to be both number and time dependent. (Table 6 and Figure 6) Calculations based on the binding of ^{125}I-BCSG with intact cells, indicated that about 750 molecules of ^{125}I-BCSG were able to bind per F745 cell whereas, AGB cells bound about 1825 molecules of ^{125}I-BCSG per cell. This was about 2.4 times more than the F745s. Thus for further investigations of BCSG membrane interaction, we employed the AGB mouse myeloma cells. It should be noted that both these cell lines bind considerably less than has been shown for our target cell line, 3T3 mouse fibroblasts. 3T3 cells have been determined to bind 1-2 X 10^4 molecules of BCSG per cell and are sensitive to BCSG effects. The reduction observed for ^{125}I-BCSG binding to F745s and AGBs may partially contribute to the refractive nature these cells exhibit toward BCSG.

EFFECTS OF ENZYMATIC DIGESTION OF CELLS ON THE ABILITY OF ^{125}I-BCSG TO BIND

The enzymatic digestions of AGBs were performed at relatively high concentrations of neuraminidase, trypsin, alpha-chymotrypsin, and proteinase K. Enzymatic digestion had dramatic effects on the ability of ^{125}I-BCSG to bind (Table 7). The effects on ^{125}I-BCSG binding to cells after treatment with trypsin was significantly decreased. Furthermore, relatively moderate decreases in binding were observed after reaction with chymotrypsin and proteinase K. Double digestion of cells with trypsin and chymotrypsin resulted

in a slight decrease in binding relative to trypsin treatment alone. Our interpretation of these data is that a membrane protein may constitute the biological receptor for BCSG.

It is interesting to note the effects on ^{125}I-BCSG binding to cells after treatment with neuraminidase. Neuraminidase digested cells showed a slight enhancement of the amount of the growth inhibitor able to bind to the cells. (Table 7) This result was not surprising since in previous studies neuraminidase liberation of N-acetyl-neuraminic acid (NANA) from eukaryotic cell surfaces has been shown to influence not only the social behavior between cells, but receptor-recognition events as well (30). Other studies (19, 20) have reported that neuraminidase converts the more complex membrane gangliosides to GM_1 and that the GM_1 structure is resistant to neuraminidase activity. In specific consideration of the former, GM_1 has been shown to enhance the growth-inhibitory effects of BCSG on some cell lines (6). However, neuraminidase hydrolyzes sialic acid from both glycolipids and glycoproteins. Therefore, an alternative explanation for the increase in ^{125}I-BCSG binding to neuraminidase-treated cells was that this digestion increased or exposed new BCSG binding sites on glycoproteins.

In summary, we have demonstrated

(1) F745 and AGB cells contain membrane gangliosides GM-1 and GD-1a.

(2) ^{125}I-BCSG was able to bind to both F745 Friend erythroleukemic and AGB mouse myeloma cells.

Calculations based on these binding data indicated that F745s contain about 750 BCSG receptors per cell and that AGBs contain approximately 1825 BCSG receptors per cell. Determination of actual receptor numbers is awaiting non-specific binding assays with BCSG.

(3) Enzymatic digestion of AGB cells with protein-specific enzymes significantly decreased 125I-BCSG bindin implicating a membrane protein involvement in BCSG-membrane interaction.

(4) Neuraminidase treatment of AG8 cells slightly enhanced 125I-BCSG binding suggesting that membrane gangliosides function in the modulation of the BCSG receptor via their mobility and head group interactions.

Table 6: 125 I - Growth Inhibitory Brain Cell Surface Glycopeptides Direct Binding to Cells -- Demonstration of Cell Number Dependence.

Cell Line	Cell Number	cpm 125 I - BCSG Bound Above Background
F745	2×10^5	887
	2×10^7	1721
AG8	2×10^5	3
	2×10^7	3019

Figure 6: Binding of 125 I - Growth-Inhibitory Brain Cell Surface Glycopeptides to F745 Friend Erythroleukemic and AG8 Myeloma Cells -- Demonstration of 125 I - BCSG Binding as a Function of Time.

Cell binding experiments were performed while holding the cells on ice. 200 microliter aliquots of the reacting suspension were removed at 0, 15, 30, 60, and 240 minutes post-125 I - BCSG addtion. The cells in each sample were pelleted and washed twice in ice-cold Hanks BBS. The amount of 125 I - BCSG bound was determined by counting gamma emission in a gamma counter. The triangles represent the results of 125 I - BCSG binding to the AG8 cells and the squares to the F745 cells.

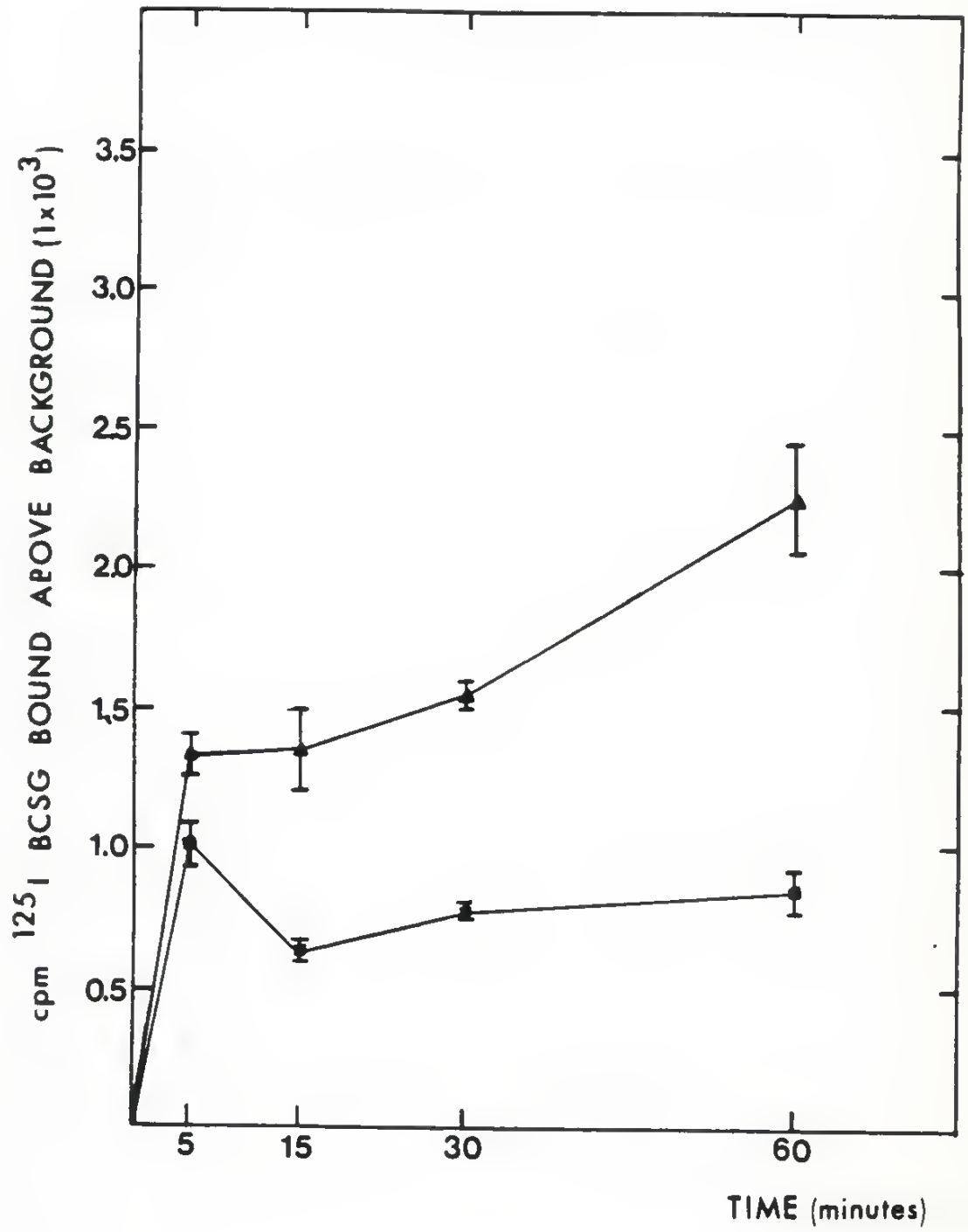
cpm ^{125}I BCSG BOUND ABOVE BACKGROUND (1×10^3)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
5
15
30
60
TIME (minutes)

Table 2: Effects of Enzymatic Digestion of AG8 Myeloma Cells on the Binding of 125 I - Growth-Inhibitory Brain Cell Surface Glycopeptides.

Enzyme	Total Enzyme Concentration	cpm 125 I - BCSG Bound Above Background
None	0.0 mg/ml	8277
Neuraminidase	0.1 unit/ml	11,147
Trypsin	1.0 mg/ml	2803
Chymotrypsin	1.0 mg/ml	6031
Trypsin (0.5mg/ml) + Chymotrypsin (0.5mg/ml)	1.0 mg/ml	2160
Proteinase K	0.25 mg/ml	3813

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Chapter IV.

FUNCTIONAL ROLE OF GM-1 GANGLIOSIDES IN THE MECHANISM OF INTERACTION OF GROWTH-INHIBITORY BRAIN CELL SURFACE GLYCOPEPTIDES WITH CELLS

INTRODUCTION

Growth of normal mammalian cells in culture is regulated in manys way, one of which is a process known as contact inhibition (1). For example, normal cells growing in a monolayer will stop growing when they come into contact with neighboring cells. This social communication between cells is exhibited by all normal cells, and is a process which is presumably lacking in cancerous or transformed cells (2). The molecular basis of this phenomenon of contact inhibition is unknown, but it is believed to occur at the level of the plasma membrane (3-5).

Cells at remote locations relative to one another are also capable of communicating. This interaction occurs via binding of extracellular substances, soluble ligands, and hormones. Hormones act as chemical messengers between cells and are capable of affecting a wide range of cellular responses involved in either long-term or short-term regulation of growth and metabolic activity (6-8).

Specific receptors on the cell surface are targets for the action of most soluble ligands and hormones (6, 8-10). The ligand-receptor complex can exert its effect on the cell by either of three known mechanisms. The first, is the internalization of the ligand-receptor complex. The second mechanism involves the activation of a membrane-bound enzyme. By the third mechanism, information is conveyed into the cell via a second messenger

system (10). Cellular communication can also be achieved through ion fluxes and pumps (11, 12). Small molecules can be transported via membrane proteins. Examples of this type of communication can be observed in neuronal or muscular membranes or at gap junctions (13).

One class of structural membrane components which are present in high concetrations in plasma membranes (14-18) and are known to participate in various cell surface recognition functions are glycosphingolipids (8, 9, 19-29). These molecules possess a hydrophilic sialic acid-containing oligosaccharide head group and are characterized by broad structural diversity (8, 9, 30). These characteristics allow gangliosides to serve as receptors for bacterial toxins (27, 31-35), plant lectins (36-38), viruses (22, 24, 39), and hormones (19, 40-43). Receptor function has been demonstrated for gangliosides naturally present in plasma membranes whether functionally incorporated into plasma membrane (40, 44), contained in liposomes (27, 40, 43), adsorbed onto plastic surfaces (45, 46), or immobilized on thin layer chromatography (TLC) plates (47). Furthermore, when added exogenously to cell cultures and incorporated into plasma membranes, glycosphingolipids inhibit cell growth and modify growth behavior (29, 48-53). However, in neoplastic cells, the composition and pattern of occurrence of these molecules changes dramatically. Glycolipids in transformed cells are less complex structurally (8). As a result, the cells lose some of their

previous cell surface recognition functions (8, 9, 28, 54).

Kinders, et al., (55, 57, 58, 61) have previously described the isolation and characterization of brain cell surface glycopeptides (BCSG) which inhibit cell growth and protein synthesis in normal (56, 57), but not in transformed cells (59). Furthermore, BCSG has been shown to bind to the cell surfaces of 3T3 mouse fibroblast cells and LM cells containing exogenously incorporated GM_1 gangliosides (62). However, even though GM_1 gangliosides are capable of sensitizing the normally BCSG-refractive LM cells (60) to the growth inhibitory effects of BCSG, they did not enhance the binding of BCSG to the LM cell surfaces (62). Additionally, Bascom, et al.,(62) have shown that GM_1 gangliosides could not bind out the inhibitory activity of BCSG in experiments where GM_1 was preincubated with BCSG prior to the treatment of 3T3 target cells.

The experiments described herein, were designed to determine whether GM_1 serves as a membrane receptor for BCSG. This experimental study was part of an investigation of BCSG-GM_1 interaction in both direct binding assays to cells and to GM_1 in various ligand-receptor binding systems. These parallel lines of investigation were employed for two reasons. First, the incorporation of exogenous gangliosides onto cell membranes from the culture medium to observe binding of BCSG to GM_1 is complicated by the presence of serum. Since a number of growth factors are present in serum and gangliosides have been shown to

interact with several of these growth factors (29) the effects of ganglioside addition to cells in culture to demonstrate BCSG binding may have been obscured. Second, the orientation of GM_1 in ligand-receptor binding assays may not have permitted binding, even by employing a variety of techniques which permit different orientations of the GM_1 molecule to make it accesible for binding by BCSG. The results obtained using GM_1 in liposome, TLC, and plastic adsorption systems are described here.

EXPERIMENTAL PROCEDURES

MATERIALS

BRAIN CELL SURFACE GLYCOPEPTIDES (BCSG)

The growth inhibitory glycopeptides were provided by Dr. T. C. Johnson (Division of Biology, Kansas State University, Manhattan, KS) (55, 61, 62)

GANGLIOISIDES

GM_1 and Bovine Brain Gangliosides (BBG) were obtained from Supelco Chemical Company, Inc., Houston, TX. They were determined by thin layer chromatography (TLC) and gas liquid chromatography (GLC) to be 98% pure and used without further purification. Tritium-labeled gangliosides were prepared according to the method prescribed by Schwarzman (63). NaB^3H_4 was obtained from New England Nuclear, Boston, MA.

CHOLERA TOXIN

Cholera toxin from Vibrio cholerae was purchased from two sources: (1) Sigma Chemical Company, St. Louis, MO; and (2) Schwarz Mann Chemical Company, Cambridge, MA. Radioiodinated cholera toxin was prepared as described by Cuatrecasas (64). ^{125}Iodine, sodium-free carrier was obtained from New England Nuclear, Boston, Massachusetts.

METHODS

THIN LAYER CHROMATOGRAPHY (TLC) BINDING ASSAY

The binding of cholera toxin to GM$_1$ gangliosides can be detected by autoradiography. ^{125}I-cholera toxin will bind to GM$_1$ on silica gel TLC plates after thin layer chromatography of the ganglioside. The technique used in this assay was essentially identical to that of Magnani, et al.,(47). This procedure is summarized in Figure 7.

LIPOSOME BINDING ASSAYS

LIPOSOME PREPARATION

Unilamellar liposomes were prepared from egg phosphatidylcholine (EPC), cholesterol, and GM$_1$ to a final mole to mole ratio of 70:30:10. Liposome constituents were mixed well, dried under N_2, and resuspended in 2.0 ml of buffer (20 mM TRIS, 0.15mM saline, 1.0% (w/v) albumin, pH 7.4). The suspension was sonicated until no

further clearing was noted and the solution was opalescent (65). An additional 5.0 ml of buffer was added post-sonication to bring the final volume of the suspension to 7.0 ml (a final phospholipid concentration of 1.0mM. Unilamellar liposomes were stored in the freezer at 0 °C until used.

Multilamellar liposomes were prepared as described by Aloj, et al., (66). Dipalmitylphosphatidylcholine (DPPC), cholesterol, and GM_1 were combined in a molar ration of 2:1:0.2 as described by Aloj, et al., (66). This procedure was a modification of an earlier protocol set forth by Kinsky, et al., (67) DPPC, cholesterol and GM_1, were dissolved in 2:1 chloroform/methanol, and combined in a 2:1:0.2 molar ratio. The preparation was mixed well, dried under a stream of N_2 and finally resuspended in a 16 x 150 mm teflon capped vial containing 2.0 ml buffer (20mM TRIS, 1mM EDTA, and 1.0% bovine serum albumin (BSA), pH 7.2). The vial was rotated for 18-24 hours at 4 °C. Five 3 mm diameter glass beads were added to the vial and the suspension was vortexed for 5-10 minutes until the lipid suspension was homogeneous. These multilamellar liposomes were washed three times with buffer and centrifuged at 7000 rpm for 10 minutes. The liposome pellet was resuspended in 2.0 ml of buffer. Fifty microliter (50 ul) aliquots of a 1:8 dilution of the final liposome solution were employed in the filtration binding assay to be described.

LIGAND-RECEPTOR BINDING ASSAYS USING RECONSTITUTED MODEL MEMBRANES

CENTRIFUGAL ELUTION ASSAY EMPLOYING MINI- GEL FILTRATION COLUMNS

We have employed a modification of the gel filtration column method published by Fry, et al., (70). The Sepharose 4B and Sephadex G-200 (Sigma Chemical Company, St. Louis, MO) gel minicolumns were prepared in a 1.0 ml plastic syringe tube which were then placed in appropriately sized centrifuge tubes. The minicolumns were centrifuged at low speed to remove the void volumes. The liposomes are loaded onto the top of the columns, allowed to absorb, and centrifuged a second time at a low g force to complete the absorption process. The determination of the time and the centrifugation speed required is dependent upon the amount and the type of gel employed.

Unilamellar liposomes, prepared as described above, were incubated on ice for 60-90 minutes with either 100,000 cpm (or about 15 ng) of ^{125}I-cholera toxin or ^{125}I-BCSG. Binding was allowed to proceed and interrupted at various time intervals. The samples were loaded onto minicolumns and allowed to enter without manipulation. Columns were spun at 100 rpm for 10 minutes to complete absorption of liposomes to the columns and then at 1000 rpm for five minutes. In the same collection tube, columns were washed twice with 100 ul of buffer and centrifuged in the same manner. The resulting eluate containinng ^{125}I-ligand bound to the unilamellar liposomes, was counted by gamma emission in a Packard Multi-Prias gamma counter. This procedure is summarized in Figure

B.

FILTRATION ASSAY FOR MULTILAMELLAR LIPOSOMES

Binding of ^{125}I-cholera toxin and ^{125}I-BCSG binding to multilamellar liposome was assayed by the filtration technique previously described by Mullin, et al., (21) and Amir, et al., (40) for thyroid plasma membranes. Multilamellar liposomes, prepared as described above, were incubated with 15 ng of ^{125}I-cholera toxin or ^{125}I-BCSG in a manner analogous to that described by Fishman, et al., (6B) for the binding of ^{125}I-choleragen to liposomes. The binding reactions were stopped after 60-90 minutes with 1.5 ml ice-cold buffer. To separate unbound ^{125}I-ligand samples were filtered under vacuum through 25 mm Millipore EGWP filters (0.2 um) (Millipore Corporation, Bedford, MASS) and washed twice with 1.0 ml buffer. Filters were dried and placed in 12 X 75 mm test tubes. The amount of ^{125}I-ligand bound to the GM$_1$ containing liposomes was determined by gamma emission counted in a Packard Multi-Prias gamma counter. This procedure is summarized in Figure 9.

GLYCOLIPID ADSORPTION ASSAY

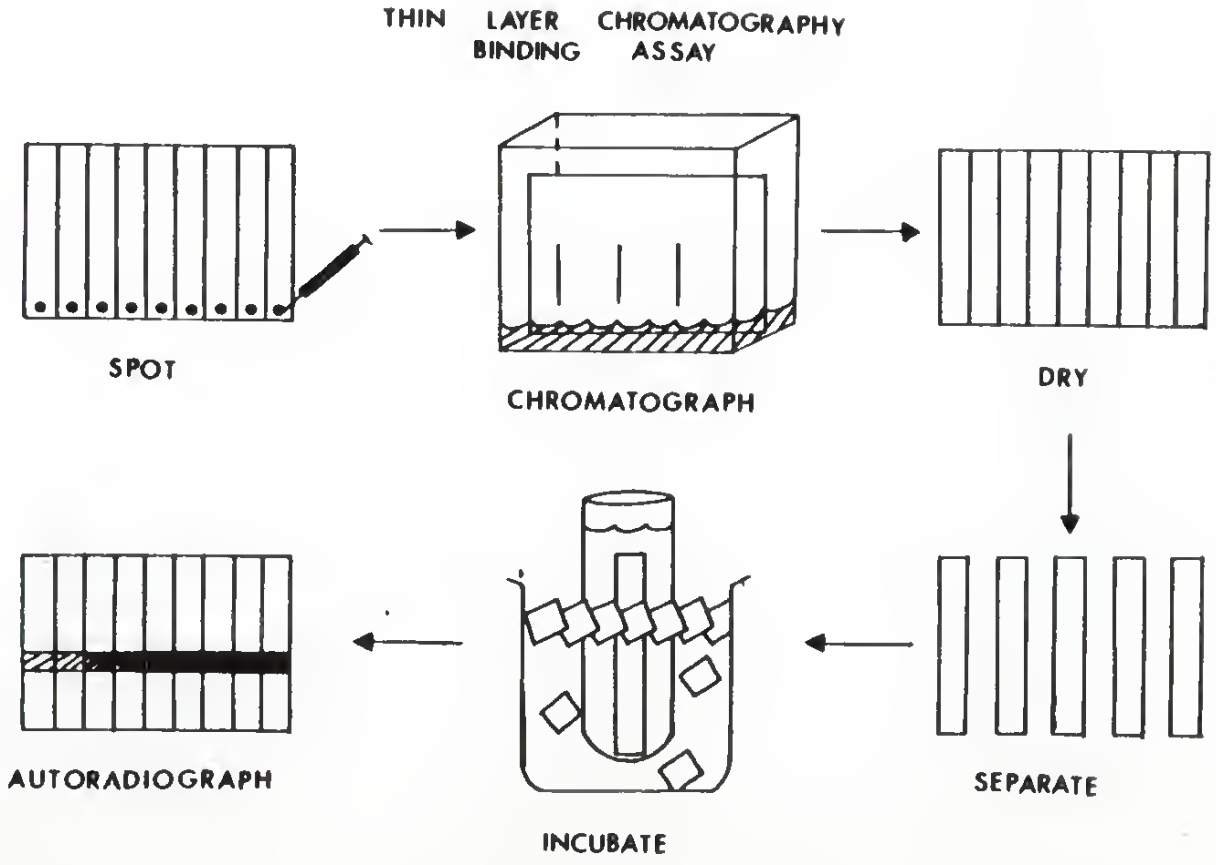
The binding of ^{125}I-BCSG to plastic adsorbed GM$_1$ gangliosides was performed similarly to the protocal of Holmgren, et al., (45, 46). This procedure is summarized in figure 10.

Figure 7: Diagrammatic representation of the thin layer chromatogrphy assay for ligand binding to gangliosides.

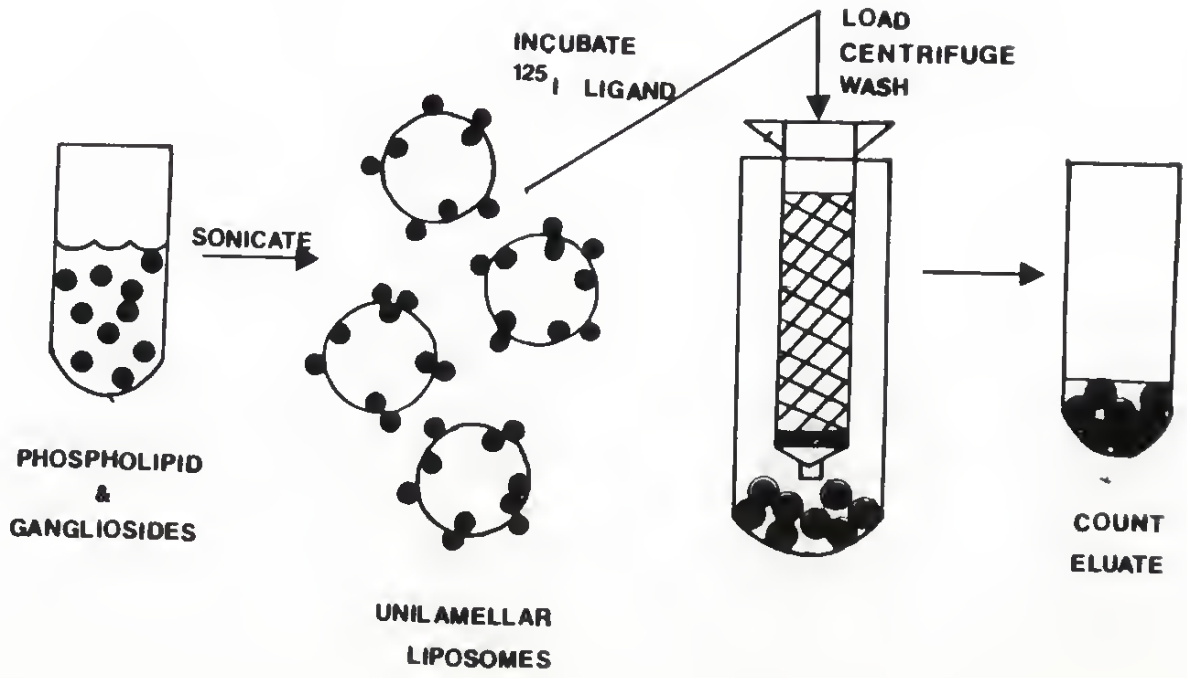
Figure 8: Diagrammatic representation of the liposome centrifugal elution assay of ligand binding to gangliosides.

Figure 9: Diagrammatic representation of the liposome filtration assay of ligand binding to gangliosides.

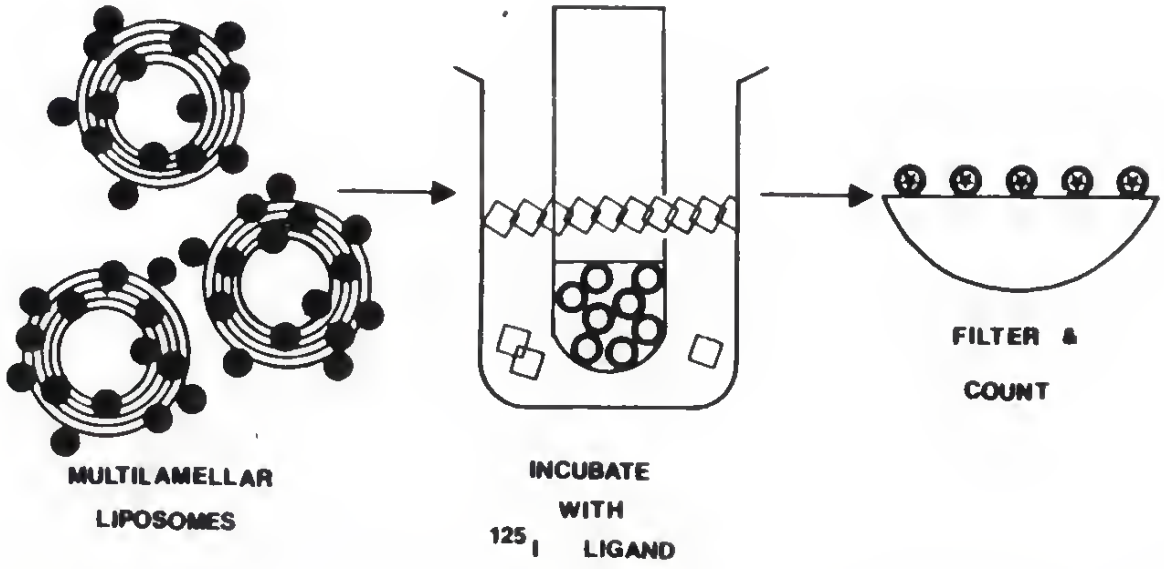
Figure 10: Diagrammatic representation of ligand binding to GM-1 gangliosides adsorbed onto polystyrene plastic tubes.


THIN LAYER CHROMATOGRAPHY
BINDING ASSAY
SPOT
CHROMATOGRAPH
DRY
SEPARATE
INCUBATE
AUTORADIOGRAPH

CENTRIFUGAL ELUTION ASSAY



FILTRATION ASSAY



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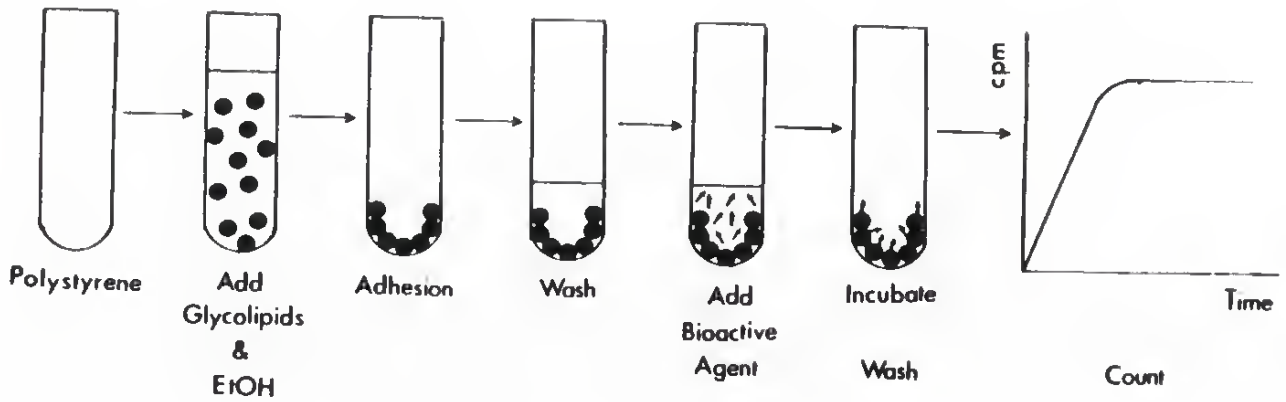

LIGAND BINDING TO IMMOBILIZED GM_1

RESULTS AND DISCUSSION

To ascertain the role of the GM_1 ganglioside as a growth regulator of the effects of BCSG on cell growth, I studied the direct binding the radioiodinated ligand to thin layer chromatographed GM_1. In initial experiments direct binding of ^{125}I-BCSG was observed after autoradiography. Similar results were obtained with the ^{125}I-cholera toxin - GM_1 ligand-receptor positive control (Figure 11). Competitive binding experiments performed between ^{125}I-BCSG and ^{125}I-cholera toxin demonstrated that the growth inhibitor competed with cholera toxin for binding GM_1 (Figure 12). Modifications now employed in the purification process of BCSG (62), a 500-fold increase in purity of the preparation of the growth inhibitor has been obtained and using the same experimental procedures these brain pool preparations of BCSG failed to yield equivalent results. Thus, the binding, of ^{125}I-BCSG to GM_1 observed in the first analyses of the TLC Binding Assay was likely due to the heterogeneity of the earliest BCSG preparations and that a "contaminating" molecular species bound to GM_1.

The inability to demonstrate BCSG binding in the same system lead to suggests that the orientation of GM_1 on the TLC plates does not to permit binding of BCSG. Furthermore, the binding affinity of BCSG to GM_1 may be so low that this system even though suitable for demonstrating ^{125}I-cholera toxin to GM_1 ligand-receptor interaction (47) cannot be employed in the same

capacity for analyzing the interaction between BCSG and GM_1. Therefore, other ligand-receptor binding assays were sought to find the most effective means to examine the potential of GM_1 as a receptor for BCSG.

Two additional direct ligand-receptor binding techniques were employed (with appropriate positive controls with cholera toxin) to investigate whether GM_1 in such systems could serve as a receptor for ^{125}I-BCSG. In liposome binding experiments, ^{125}I-BCSG was not observed to bind to GM_1 incorporated into either unilamellar or multilamellar liposomes. The presence or absence of GM_1 or cholesterol, the species of phosphatidylcholine, the ratio of phospholipid to cholesterol to GM_1, and the means by which bound versus free ^{125}I-ligand were separated (that is, mini-gel column filtration or vacuum filtration) had no effect on the results. (Tables 8-11 and Figures 13, 14) The experimental recovery of liposomes containing ^{3}H-cholesterol was approximately 89.8% regardless of the liposome sample (50-200 ul) (Tables 12 and 13) (This value is comparable to that obtained by Fishman, *et al.*, (68) who reported the recovery of liposomes by similar techniques as 87.6%.).

In the third ligand-receptor binding assay employed to demonstrate GM_1 serves a receptor function for BCSG, ^{125}I-BCSG did not bind to GM_1 adsorbed onto polystyrene tubes (Table 14). Quantitation of ganglioside adsorption was determined by incorporating 10 nmoles of ^{3}H-GM_1 into the total 60 nmoles of

ganglioside used for the adsorption procedure. Approximately 25% of the ^{3}H-GM$_1$ was adsorbed at 18-24 hours and this amount remained unchanged for 72 hours after adsorption (Data not shown). Thus each reaction vessel contained about 15 nanomoles of GM$_1$ (or 2 X 10^{12} molecules of GM$_1$). The addition of 0.01% Tween 20 to the buffer did not cause solubilization of the adhered gangliosides, even at extended periods of incubation (Data not shown). Furthermore, the polystyrene-adsorbed GM$_1$ retained its specific ligand-binding properties as demonstrated by its ability to bind ^{125}I-cholera toxin. It has been reported (46) that the attachment of the GM$_1$ ganglioside to polystyrene plastic exhibits a log-linear relationship between the amount of GM$_1$ bound and time. Holmgren, *et al.*, (45) further demonstrated a similar linear relationship between the amount of ^{125}I--cholera toxin able to bind to plastic-adsorbed GM$_1$. The applicability of this binding system has been extended to viruses and ganglioside receptor analyses (45, 46). However, we could not demonstrate that ^{125}I-BCSG binds to GM$_1$ using this assay.

From these data it was concluded that GM$_1$ in direct ligand-receptor binding assays did not bind to ^{125}I-BCSG (Data summarized in Table 15). These results correlate directly with data obtained in our cell binding experiments (See Chapter 3, and ref. 62). These analyses imply that GM$_1$ does not serve as a high affinity membrane receptor for the growth inhibitory glycopeptides.

It has been demonstrated that various growth factors are affected by the presence of gangliosides (29, 69). The incorporation of exogenous GM_1 gangliosides has been demonstrated to inhibit the effects of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), but in neither case has PDGF or EGF been demonstrated to bind to the gangliosides. (70) On the other hand, the GM_3 ganglioside has been shown to enhance the sensitivity of cells to fibroblast growth factor (FGF). However, FGF has also does not bind to GM_3. Furthermore, GM_1 has been shown to modulate the PDGF receptor by altering its ability to be phosphorylated (69). It is possible we may be analyzing a system analogous has been described for these other growth factors.

Figure 11: 125 I - Ligand Binding to GM-1 Gangliosides Chromatographed on Thin Layer Chromatography (TLC) plates and visualized by autoradiography.

40 nanomoles of GM-1 ganglioside were chromatographed on Kodak Eastman silica gel TLC plates in a solvent system composed of chloroform/methanol/magnesium chloride/ammonium hydroxide (60:35:7.5:3). Once the solvent had evaporated from the plates, direct binding of 125 I - Cholera Toxin or 125 I - BCSG was performed on ice in a TLC binding buffer containing: 0.15mM NaCl; 10mM Na_2HPO_4; and 1.0% polyvinyl pyrrolidone at pH 7.4. After incubation the strips from the plates were washed twice with cold buffer, dried, and autoradiographed. Figure 10 illustrates a typical autoradiographed TLC plate after direct binding of 125 I - cholera toxin.

Figure 12: Competitive Binding For GM-1 By 125 I - Cholera Toxin and Growth-Inhibitory Brain Cell Surface Glycopeptides on Thin Layer Chromatography Plates.

40 nanomoles of GM-1 ganglioside were chromatographed on Kodac Eastman silica gel TLC plates in a solvent system of chloroform/methanol/magnesium chloride/ammonium hydroxide (60:35:7.5:3). After the solvent had evaporated from the plates and the plates prepared for the direct binding of 125 I - Cholera Toxin to GM-1, incubation buffers of the appropriate tubes were prepared containing increasing concentrations of the Brain Cell Surface Glycopeptides. Addition of up to 1.75 ug/ml BCSG to the tubes was able to decrease the amount of 125 I - Cholera Toxin to bind to the GM-1 on the TLC plates by over 2000 counts per minute bound above background.

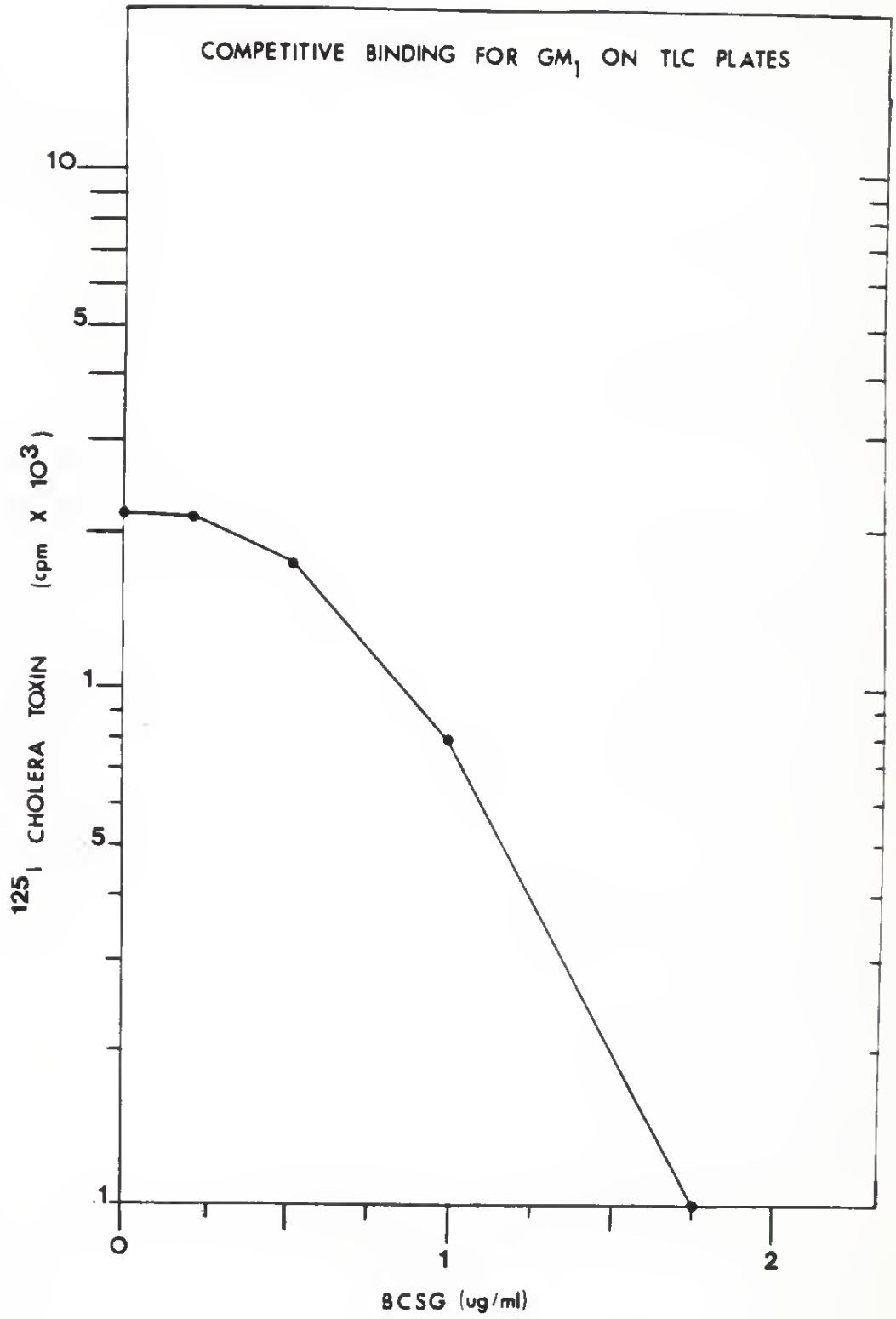
COMPETITIVE BINDING FOR GM1 ON TLC PLATES
10
5
1
5
.1
125I CHOLERA TOXIN (cpm X 10^3)
0
1
2
BCSG (ug/ml)

Table 8: Specific Binding of 125 I - Cholera Toxin and 125 I - Growth-Inhibitory Brain Cell Surface Glycopeptides to Unilamellar Liposomes via the Liposome Centrifugal Elution Assay with Sepharose 4B Syringe Minicolumns.

125 I - Ligand	Liposome Composition	cpm 125 I Bound	% Control Eluate	Total % Bound
125 I - cholera toxin	EPC/CHOL	9617	100%	5%
	EPC/CHOL/BBG	21213	87%	11%
	EPC/CHOL/ SULFATIDES	29783	81%	15.5%
125 I - BCSG	EPC/CHOL	661	100%	2.9%
	EPC/CHOL/BBG	1122	92%	4.9%
	EPC/CHOL/ SULFATIDES	905	94%%	3.9%

Table 2: Time dependence of 125 I - Growth-Inhibitory Brain Cell Surface Glycopeptides Binding to Unilamellar Liposomes via the Liposome Centrifugal Elution Assay Employing Sepharose 4B Syringe Minicolumns.

Length of Incubation (minutes)	Liposome Composition	cpm 125 I Eluted (% 125 I recovered)	Total cpm Bound Assay #1	Assay #2
0	EPC/CHOL (70:30)	15,571 (74.2%)	3250	4691
30		17257 (82.2%)	2902	4596
60		17510 (83.4%)	3675	4376
0	EPC/CHOL/BBG (70:30:5)	14609 (69.6%)	2343	3739
30		17558 (83.7%)	3795	4729
60		16615 (79.2%)	1959	4731

Figure 13: Time Dependence of 125 I - Cholera Toxin Binding to Egg Phosphatidylcholine (EPC) and Cholesterol (CHOL) Unilamellar Liposomes With and Without Incorporated GM-1 Gangliosides via the Liposome Centrifugal Elution Assay Employing Sephadex G-200 Syringe Minicolumns.

EPC/CHOL (70:30, mole/mole) and EPC/CHOL/GM-1 (70:30:10, mole/mole) unilamellar liposomes were prepared as described in the experimental procedures. These liposomes were incubated in PBS with 100,000 cpm 125 I - cholera toxin for 15, 30, and 60 minutes. Unbound 125 I - cholera toxin was separated from that bound to the liposomes by centrifuging at a low g force (1000rpm) through 1.0 ml sepharose 4B mini - gel filtration columns (70). The open circles represent the amount of 125 I - cholera toxin bound to EPC/CHOL liposomes and the closed circles represent the amount of 125 I - cholera toxin bound to EPC/CHOL/GM-1 liposomes.

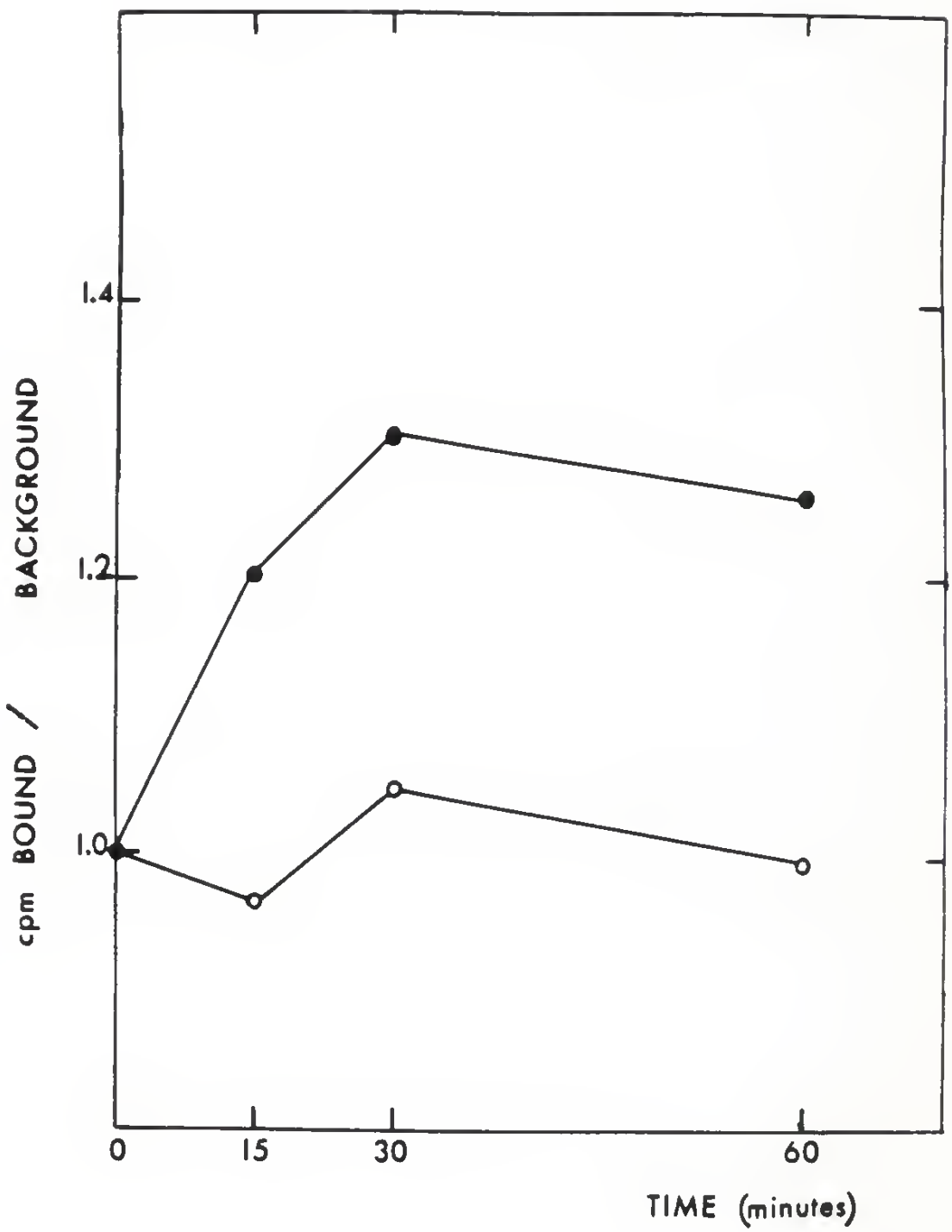
1.4
1.2
1.0
cpm BOUND / BACKGROUND
0
15
30
60
TIME (minutes)

Table 10: Specific Binding of 125 I - Cholera Toxin to Unilamellar Liposomes via the Liposome Centrifugal Elution Assay Employing Sephadex G-200 Syringe Minicolumns.

Liposome Composition	cpm 125 I - Cholera Toxin Bound	% Control Bound	Total % Bound
Experiment 1:			
EPC (100)	15,350	100%	7.9%
EPC/CHOL (70:30)	16,188	86.7%	10.2%
EPC/CHOL/BBG (70:30:5)	26,574	122%	16.7%
Experiment 2:			
DPPC/CHOL (70:30)	232	100%	4.8%
DPPC/CHOL/GM-1	543	200%	21.8%

Table 11: 125 I - Growth-Inhibitory Brain Cell Surface Glycopeptides Binding to Multilamellar Liposomes Containing Bovine Brain Gangliosides. Separation of Bound versus Free 125 I - BCSG via the Liposome Filtration Assay.

Liposome Composition	cpm 125 I - BCSG Bound	Total % Bound
DPPC/CHOL (70:30)	235	4.7%
DPPC/CHOL/BBG (70:30:5)	210	4.2%

Figure 14: Time Dependence of 125 I - Cholera Toxin and 125 I - Growth-Inhibitory Brain Cell Surface Glycopeptides (BCSG) Binding to Dipalmitoylphosphatidylcholine (DPPC) Cholesterol (CHOL) and GM-1 Gangliosides (70:30:5) Multilamellar Liposomes via the Liposome Filtration Assay.

DPPC/CHOL/GM-1 (70:30:5, mole/mole) and DPPC/CHOL (70:30, mole/mole) unilamellar liposomes were prepared as described in the experimental procedures. These liposomes were incubated in buffer (20 mM TRIS, 0.15 mM EDTA, 1.0% BSA, pH 7.2) containing either 100,000 cpm 125 I -cholera toxin or 100,000 cpm 125 I - BCSG for 30 and 60 minutes. Unbound 125 I - ligand was separated from that bound to the liposomes by centrifuging at a low g force (1000 rpm) through 1.0 ml sephadex G-200 mini - gel filtration columns (70). The amount of 125 I - ligand bound to DPPC/CHOL liposomes was subtracted from that bound to DPPC/CHOL/GM-1 liposomes as background. The closed circles represent the amount of 125 I - cholera toxin bound to the GM-1 in the DPPC/CHOL/GM-1 liposomes and the closed squares represent the amount of 125 I - BCSG bound to the GM-1 in similarly prepared DPPC/CHOL/GM-1 liposomes.

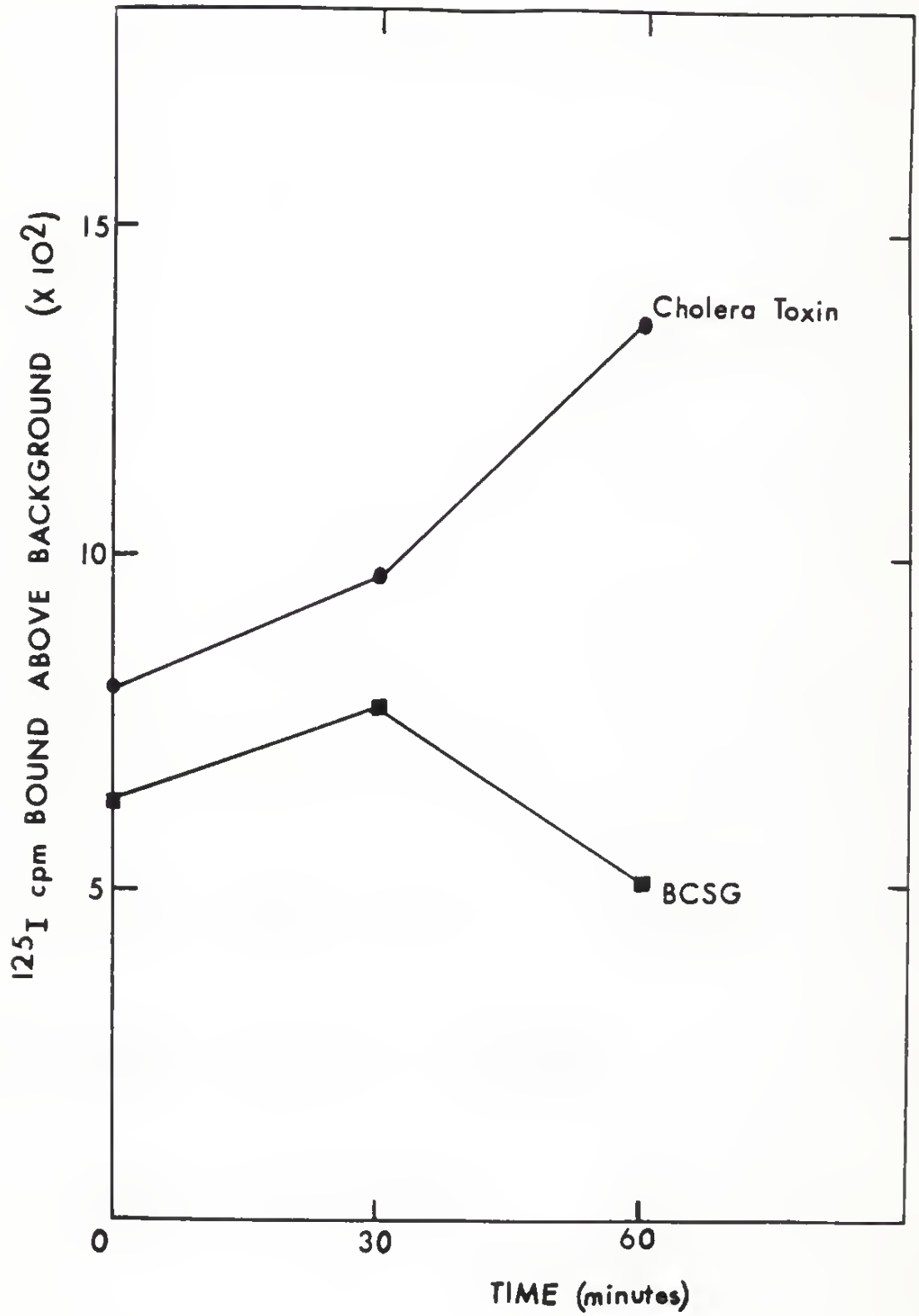
15
10
5
0
30
60
Cholera Toxin
BCSG
^{125}I cpm BOUND ABOVE BACKGROUND (X 10^2)
TIME (minutes)

Table 12: Percent Recovery of 3 H - Labeled Liposomes Through Sepharose Minicolumns via the Liposome Centrifugal Elution Assay.

Minicolumn Bed Volume (cc)	Type of Syringe Plug Employed	Length of Spin (minutes)	% 3 H - Liposomes Recovered (in 1200 ul)	
4B Separose:				
0.73	Cellophane	3	70.4%	
0.60	Cellophane	1	77.9%	
0.75	Spun Glass	3	76.6%	
0.65	Spun Glass	1	83.2%	*
6B Sepharose:				
0.84	Cellophane	3	77.2%	
0.77	Cellophane	1	79.9%	
0.78	Spun Glass	3	79.4%	
0.76	Spun Glass	1	81.6%	

* = Syringe Minicolumn Method Employed.

Table 13: Recovery of 3 H - Liposomes versus 125 I - Cholera Toxin Through Sephadex G-200 Minicolumns -- Determination of Retention Times of Liposomes versus Free 125 I - Ligand.

Substance Recovered	Eluate	% cpm Recovered 200 microliter wash #1	#2	#3	#4	#5	Total % Recovered
3 H - Liposomes	55.0 (55.0)	27.0 (82.0)	5.6 (87.6)	1.8 (89.4)	1.2 (90.6)	1.0 (91.6)	91.6%
125 I - Cholera Toxin	7.0 (7.0)	10.3 (17.3)	14.0 (31.3)	14.3 (45.6)	12.4 (58.0)	10.5 (68.5)	68.5%

Table 14: Binding of 125 I - Growth-Inhibitory Brain Cell Surface Glycopeptides to GM-1 Gangliosides Adsorbed onto Polystyrene Tubes.

Radioiodinated Ligand	Nanomoles of GM-1 Ganglioside	cpm 125 I - Ligand Bound Length of Incubation (Hours) 0.0	2.0
125 I - BCSG	0.0	203 +/- 237 (0.51%)	217 +/- 174 (0.49%)
	0.2	226 +/- 172 (0.53%)	300 +/- 225 (0.67%)

Figure 15: Time Dependence of Binding of 125 I - Cholera Toxin to Bovine Brain Gangliosides (BBG) Adsorbed onto Polystyrene Plastic Test Tubes.

60 nanomoles of BBG (containing approximately 40% GM-1 gangliosides) were adsorbed onto 12 X 75 mm Falcon polystyrene tubes (Becton and Dickinson) by the ethanol evaporation method of Holmgren, et al., (45, 46). In a 200 ul total volume, composed of buffer (20 mM TRIS, 0.15 mM EDTA, 0.01% Tween 20, pH 7.2) and 50,000 cpm (about 7.5 ng) of 125 I - cholera toxin, the adsorbed gangliosides were incubated with 125 I - ligand for 15, 30, and 60 minutes at room temperature. Unbound 125 I - ligand was aspirated from the tubes and the tubes were washed with 200 ul of buffer. The amount of 125 I - cholera toxin bound to polystyrene tubes containing no gangliosides and treated in a similar manner as tubes with adsorbed gangliosides were subtracted from the amount of 125 I -cholera toxin bound to the plastic adsorbed gangliosides as background.

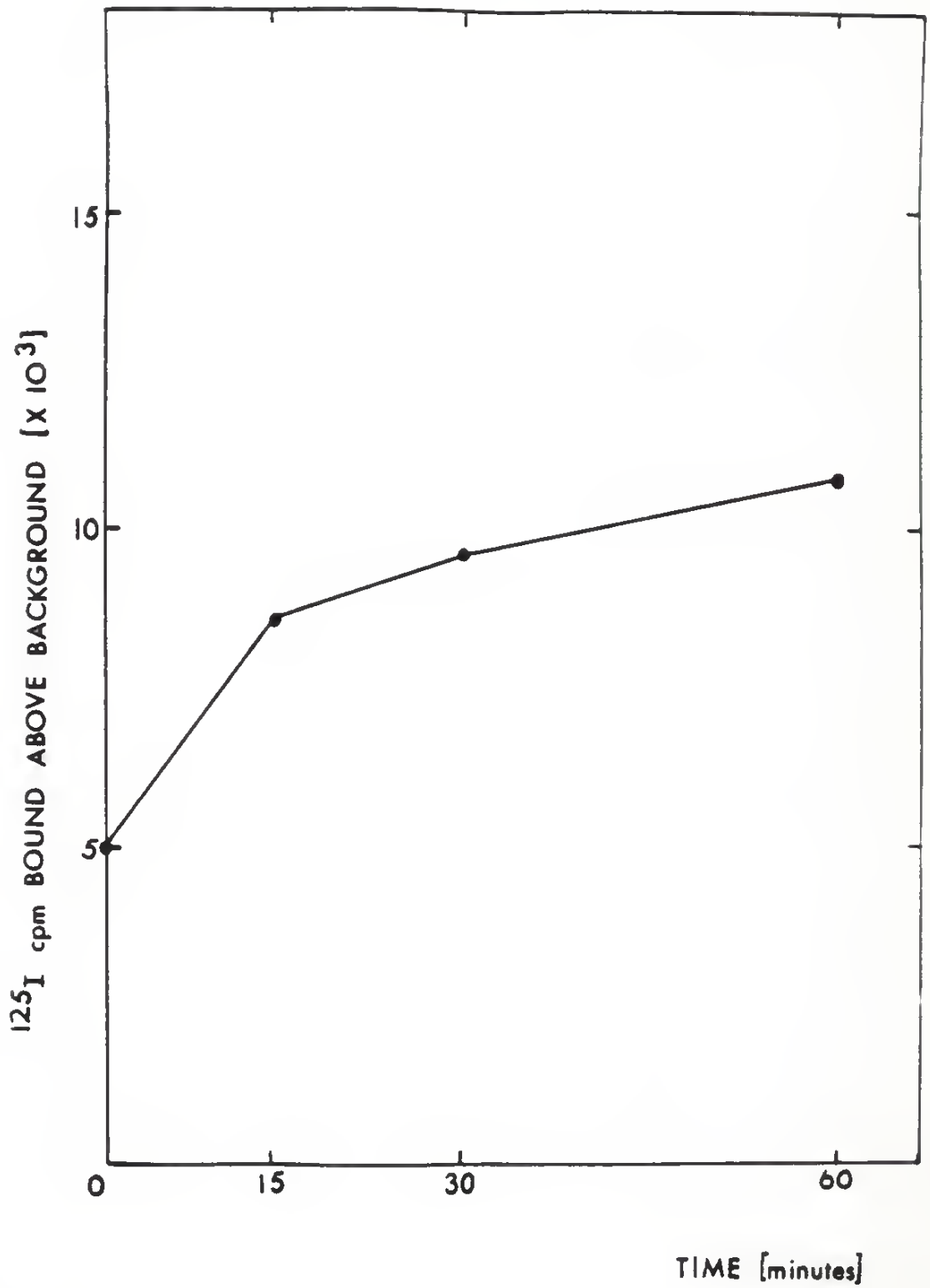
15
10
5
0
15
30
60
^{125}I cpm BOUND ABOVE BACKGROUND [X 10^3]
TIME [minutes]

Table 15: Data Summary of 125 I - Ligand Binding to the GM-1 Ganglioside via Various *in vitro* Binding Assays.

Assay Method	125 I - Cholera Toxin	125 I - BCSG
TLC Binding Assay		
125 I - BCSG	+	+/-
Competition Between Cold BCSG and 125 I - Cholera Toxin	+	+/-
Liposome Binding Systems		
Centrifugal Elution Assay	+	-
Filtration Assay	+	-
Glycolipid Adsorption Assay	+	-

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MEMBRANE DYNAMICS OF THE GM$_1$ GANGLIOSIDE -- CHARACTERIAZATION OF THE FUNCTIONAL ROLE OF GM$_1$ IN GROWTH REGULATION AND LIGAND-RECEPTOR INTERACTIONS ON LIPID MOBILITY

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

As plasma membrane components of many cells, gangliosides have been shown to serve as membrane receptors for various biologically active substances. Furthermore, gangliosides have been demonstrated to modulate the effects of various growth factors. Even though gangliosides are capable of modulating the effects of growth factors such as platelet-derived growth factor and epidermal growth factor, they do not bind to these growth-affecting molecules. We have examined the interaction of gangliosides with molecules which inhibit the growth of cultured animal cells.

Growth-inhibitory glycopeptides have been purified and characterized from bovine cerebral cortex cells. These glycopeptides are capable of inhibiting both protein synthesis and cell division in normal cells. Mouse LM cells have no detectable gangliosides within their plasma membranes and are refractory to the inhibitor. Incubation of exogenous GM_1 gangliosides with the LM cells confers sensitivity to the inhibitor upon the cells. The sensitization of cells to the inhibitory action of the glycopeptides implies that GM_1 may act as a membrane receptor or receptor modulator for these molecules. Elucidation of the functional role played by GM_1 in this growth-regulatory process was analyzed by both *in vivo* and *in vitro* ligand-receptor binding assays to detect binding of inhibitor to gangliosides. In cell binding assays, it was shown that incubation of exogenous gangliosides with these cells, though capable of sensitizing them

to the growth inhibitor, did not result in an increase in binding of the inhibitor to the cells. Furthermore, various *in vitro* ligand-receptor binding assays were performed using the cholera toxin-GM_1 ligand receptor model. These studies also demonstrated the inability of the growth inhibitor to bind to GM_1 gangliosides. In addition, protease digestion of cells able to bind the growth inhibitor significantly decreased inhibitor binding. This implies that a membrane protein was involved in the growth inhibitor-membrane interaction. Neuraminidase treatment of the same cells was able to enhance slightly the binding of the growth-inhibitory glycopeptides to the cells apparently from an increase in membrane GM_1. These data suggest that membrane ganglioside GM_1 functions in the modulation of the actual membrane receptor for the growth inhibitory glycopeptides.

Hypotheses explaining the molecular mechanism of GM_1 as a modulator of membrane receptor(s) are speculative at best. Progress in the elucidation of the role of gangliosides as membrane receptors has been achieved through studies of the interaction of the enterotoxin of *Vibrio cholerae* with the GM_1 ganglioside. As a lectin-like molecule, cholera toxin has proved to be a useful investigative tool towards the understanding of membrane structure and receptor mobility as well as receptor function. GM_1 ganglioside has been demonstrated to be freely mobile within the lipid bilayer. Thus, GM_1 is capable of interacting with other membrane components such as membrane proteins (receptors) and lipids. Specifically, when GM_1 is

incorporated into model membrane lipid systems, the interaction of GM_1 with the phospholipid(s) results in an increase in membrane order. We examined the effects of ligand-receptor interaction between GM_1 and cholera toxin to elucidate the functional and behavioral role of GM_1 as a membrane receptor. By employing fluorescence polarization and light scattering, we were able to detect lectin-induced agglutination of liposomes containing GM_1. In addition, glycolipid clustering events within these model membranes resulted in a disordering of the membrane lipids. Such events can significantly alter membrane dynamics which in turn can lead to changes in membrane permeability or cell surface function.